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(54) Title: VACCINE (57) Abstract The present invention provides Human Papilloma Virus (HPV) fusion proteins, linked to an immunological fusion partner that provides T helper epitopes to the HPV antigen. Vaccine formulations are provided that are useful in the treatment or Prophylaxis of HPV induced tumours.		

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VACCINE

The present invention relates to vaccine compositions, comprising an E6 or/ and E7 or E6, E7 fusion protein from an HPV strain optionally linked with an
5 immunological fusion partner and formulated with a CpG containing oligonucleotide into vaccines that find utility in the treatment or prophylaxis of human papilloma virus induced tumours or lesions. In particular, the present invention relates to vaccines comprising fusions proteins, comprising a protein or part of a protein that provides T helper epitopes (such as protein D from Haemophilus influenzae B) and an antigen
10 from a human-papilloma virus (eg comprising an E6 or E7 protein from HPV 16 or 18 strain associated with cancer) that find utility in the treatment or prophylaxis of human papilloma induced tumours, wherein the vaccine is formulated with a CpG containing oligonucleotide as an adjuvant.

Papillomaviruses are small naked DNA tumour viruses (7.9 kilobases, double
15 strand), which are highly species-specific. Over 70 individual human papillomavirus (HPV) genotypes have been described. Papillomaviruses are classified on the basis of species of origin (human, bovine etc.) and of the degree of genetic relatedness with other papillomaviruses from the same species. HPVs are generally specific for the skin or mucosal surfaces and have been broadly classified into "low" and "high" risk
20 viruses.

Low risk HPVs usually cause benign *lesions* (warts or papillomas) that persist for several months or years. High risk HPVs are associated with pre-neoplastic lesions and cancer. The strongest positive association between an HPV virus and human cancer is that which exist between HPV 16 and 18 and cervical carcinoma.
25 More than ten other HPV types have also been found in cervical carcinomas including HPV 31 and HPV 33 although at less frequency.

Genital HPV infection in young sexually active women is common and most individuals either clear the infection, or if lesions develop, these regress. Only a subset of infected individuals has lesions which progress to high grade intraepithelial
30 neoplasia and only a fraction of these progress further to invasive carcinoma.

The molecular events leading to HPV infection have not been clearly established. The lack of an adequate *in vitro* system to propagate human

papillomaviruses has hampered the progress to a best information about the viral cycle.

Today, the different types of HPVs have been isolated and characterised with the help of cloning systems in bacteria and more recently by PCR amplification. The molecular organisation of the HPV genomes has been defined on a comparative basis with that of the well characterised bovine papillomavirus type 1 (BPV1).

Although minor variations do occur, all HPVs genomes described have at least seven early genes, E1 to E7 and two late genes L1 and L2. In addition, an upstream regulatory region harbors the regulatory sequences which appears to control most transcriptional events of the HPV genome.

E1 and E2 genes are involved in viral replication and transcriptional control, respectively and tend to be disrupted by viral integration. E6 and E7 are involved in viral transformation. E5 has also been implicated in this process.

In the HPVs involved in cervical carcinoma such as HPV 16 and 18, the oncogenic process starts after integration of viral DNA. The integration results in the inactivation of genes coding for the capsid proteins L1 and L2 and loss of E2 repressor function leads to deregulation of the E6/E7 open reading frame installing continuously overexpression of the two early proteins E6 and E7 that will lead to gradually loss of the normal cellular differentiation and the development of the carcinoma. E6 and E7 overcome normal cell cycle by inactivating major tumor suppressor proteins, p53 and pRB, the retinoblastoma gene product, respectively.

Carcinoma of the cervix is common in women and develops through a pre-cancerous intermediate stage to the invasive carcinoma which frequently leads to death. The intermediate stages of the disease is known as cervical intraepithelial neoplasia and is graded I to III in terms of increasing severity (*CIN I-III*).

Clinically, HPV infection of the female anogenital tract manifests as cervical flat condylomas, the hallmark of which is the koilocytosis affecting predominantly the superficial and intermediate cells of the cervical squamous epithelium.

Koilocytes which are the consequence of a cytopathic effect of the virus, appear as multinucleated cells with a perinuclear clear haloe. The epithelium is thickened with abnormal keratinisation responsible for the warty appearance of the lesion.

Such flat condylomas when positive for the HPV 16 or 18 serotypes, are high-risk factors for the evolution toward cervical intraepithelial neoplasia (CIN) and carcinoma in situ (CIS) which are themselves regarded as precursor lesions of invasive cervix carcinoma.

5 The natural history of oncogenic HPV infection presents three consecutive phases, namely:

- (1) a latent infection phase,
- (2) a phase of intranuclear viral replication with product of complete virions, which corresponds to the occurrence of koilocytes. At this stage, the HPV is producing its
10 full range of proteins including E2, E5, E6, E7, L1 and L2.
- (3) a phase of viral integration into the cellular genome, which triggers the onset of malignant transformation, and corresponds to CIN II and CIN III/CIS with progressive disappearance of koilocytes. At this stage, the expression of E2 is down-regulated, the expression of E6 and E7 is enhanced. Between CIN II/III and CIN III /
15 Cervix carcinoma the viral DNA changes from being episomal in the basal cells to integration of E6 and E7 genes only (tumoral cells). 85% of all cervix carcinomas are squamous cell carcinomas most predominantly related to the HPV16 serotype. 10% and 5% are adenocarcinomas and adenosquamous cell carcinomas respectively, and both types are predominantly related to HPV 18 serotype. Nevertheless other
20 oncogenic HPV's exist.

International Patent Application No. WO 96/19496 discloses variants of human papilloma virus E6 and E7 proteins, particularly fusion proteins of E6/E7 with a deletion in both the E6 and E7 proteins. These deletion fusion proteins are said to be immunogenic.

25 Immunomodulatory oligonucleotides contain unmethylated CpG dinucleotides ("CpG") and are known (WO 96/02555, EP 468520). CpG is an abbreviation for cytosine-guanosine dinucleotide motifs present in DNA. Historically, it was observed that the DNA fraction of BCG could exert an anti-tumour effect. In further studies, synthetic oligonucleotides derived from BCG gene sequences were shown to be
30 capable of inducing immunostimulatory effects (both in vitro and in vivo). The authors of these studies concluded that certain palindromic sequences, including a central CG motif, carried this activity. The central role of the CG motif in

immunostimulation was later elucidated in a publication by Krieg, Nature 374, p546 1995. Detailed analysis has shown that the CG motif has to be in a certain sequence context, and that such sequences are common in bacterial DNA but are rare in vertebrate DNA.

5 It is currently believed that this evolutionary difference allows the vertebrate immune system to detect the presence of bacterial DNA (as occurring during an infection) leading consequently to the stimulation of the immune system. The immunostimulatory sequence as defined by Krieg is:

Purine Purine CG pyrimidine pyrimidine and where the CG motif is not
10 methylated. In certain combinations of the six nucleotides a palindromic sequence is present. Several of these motifs, either as repeats of one motif or a combination of different motifs, can be present in the same oligonucleotide. The presence of one or more of these immunostimulatory sequence containing oligonucleotides can activate various immune subsets, including natural killer cells (which produce interferon γ and
15 have cytolytic activity) and macrophages (Wooldrige et al Vol 89 (no. 8), 1977). Although other unmethylated CpG containing sequences not having this consensus sequence have now been shown to be immunomodulatory.

The present invention provides compositions comprising either an E6 or/and E7 or an E6/E7 fusion protein optionally linked to an immunological fusion partner
20 having T cell epitopes, and adjuvanted with an immunomodulatory CpG containing oligonucleotide.

In a preferred form of the invention, the immunological fusion partner is derived from protein D of Hemophilus influenza B. Preferably the protein D derivative comprises approximately the first 1/3 of the protein, in particular
25 approximately the first N-terminal 100-110 amino acids. The protein D may be lipidated (Lipo Protein D). Other immunological fusion partners include the non-structural protein from influenzae virus, NS1 (hemagglutinin). Typically the N terminal 81 amino acids are utilised, although different fragments may be used provided they include T-helper epitopes.

30 In another embodiment the immunological fusion partner is the protein known as LYTA. Preferably the C terminal portion of the molecule is used. Lyta is derived from Streptococcus pneumoniae which synthesize an N-acetyl-L-alanine amidase,

amidase LYTA, (coded by the *lytA* gen {Gene, 43 (1986) page 265-272} an autolysin that specifically degrades certain bonds in the peptidoglycan backbone. The C-terminal domain of the LYTA protein is responsible for the affinity to the choline or to some choline analogues such as DEAE. This property has been exploited for the development of E.coli C-LYTA expressing plasmids useful for expression of fusion proteins. Purification of hybrid proteins containing the C-LYTA fragment at its amino terminus has been described {Biotechnology: 10, (1992) page 795-798}. As used herein a preferred embodiment utilises the repeat portion of the Lyta molecule found in the C terminal end starting at residue 178. A particularly preferred form incorporates residues 188 - 305.

Accordingly, the present invention in preferred embodiment provides compositions comprising an immunomodulatory CpG oligonucleotide and a fusion proteins comprising Protein D - E6 from HPV 16, Protein D - E7 from HPV 16 Protein D - E7 from HPV 18, Protein D - E6 from HPV 18, and Protein D E6 E7 from both HPV 16 and 18. The protein D part preferably comprises the first 1/3 of protein D. It will be appreciated that other E6 and E7 proteins may be utilised from other HPV subtypes.

The proteins utilised in the present invention preferably are expressed in E. coli. In a preferred embodiment the proteins are expressed with a Histidine tail comprising between 5 to 9 and preferably six Histidine residues. These are advantageous in aiding purification.

The protein E7 may in a preferred embodiment carry one or several mutations in the binding site for the *rb* (retinoblastoma gene product) and hence eliminate any potential transforming capacity. Preferred mutations for HPV 16 E7 involve replacing Cys₂₄ with Glycine, or Glutamic acid₂₆ with Glutamine. In a preferred embodiment the E7 protein contains both these mutations.

Preferred mutations for the HPV 18 E₇ involve replacing Cys₂₇ with Glycine and/or Glutamic acid₂₉ with Glutamine. Again preferably both mutations are present.

Single or double mutations may also be introduced p53 region of E₆ to eliminate any potential transforming ability.

In a further embodiment of the invention there is provided an E6 E7 fusion protein from HPV linked to an immunological fusion partner and a CpG immunomodulatory oligonucleotide.

The vaccine of the present invention preferentially induces a TH1 immune response.

Two main types of Helper T cells have been characterized TH1 and TH2, which differ in the type of cytokines they secrete. These cytokines can be considered as the driving force behind the development of 2 different types of immune response : TH1-type of immune response is associated with cell mediated effector mechanisms such as production of the INF- γ and IL-2 cytokines by T-lymphocytes. INF- γ which in turn can activate other cells and induce them to secrete other important cytokines and mediators (INF- γ - activated NK cells produce IL12, IL2-activated NK cells are transformed into lymphokine activated killer cell (LAK), INF- γ -activated macrophages secrete inflammatory mediators like TNF α , IL1, IL6 and release nitric oxide, IL2 can provide help for the differentiation of antigen specific, haplotype restricted cytotoxic T lymphocytes (CTL). At the antibody level, in mice, Th1-type of immune response is also associated with the generation of antibodies of the IgG2 isotype (IgG2a in Balb/c mice and IgG2b in C57BL/6 mice).

The Th2-type of immune response is associated with a humoral immune response to the antigen, with the production of cytokines like IL4, IL5, IL6, IL10 and by the generation of a broad range of immunoglobulin isotypes including in mice IgG1, IgA, and IgM.

In man the distinction of Th1 and Th2-type immune responses is not absolute. An individual will support an immune response which is predominantly Th1 or predominantly Th2. However, it is often convenient to consider the families of cytokines in terms of that described in murine CD4 +ve T cell clones by Mosmann and Coffman (*Mosmann, T.R. and Coffman, R.L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual Review of Immunology, 7, p145-173*).

In the human TH1 type of response is also associated with the presence of cytokine (IFN γ and IL2) eventually with the presence of CTL and IgG2 isotypes in mice correspond to IgG1 type antibodies

This type 1 phenotype is of particular importance in protecting against viral and intracellular bacterial infections as well as in the treatment of cancer.

To manufacture the proteins used in the invention by recombinant techniques, an expression strategy can be used which involves fusion of E7, E6 or E6/E7 fusion to the 1/3-N-terminal portion of protein D from Haemophilus influenzae B, an immunological fusion partner providing T cell helper epitopes. An affinity polyhistidine tail is engineered at the carboxy terminus of the fusion protein allowing for simplified purification. Such recombinant antigen is overexpressed in *E. coli* as insoluble protein.

10 The proteins of the invention may be coexpressed with thioredoxin in trans (TIT). Coexpression of thioredoxin in trans versus in cis is preferred to keep antigen free of thioredoxin without the need for protease. Thioredoxin coexpression eases the solubilisation of the proteins of the invention. Thioredoxin coexpression has also a significant impact on protein purification yield, on purified-protein solubility and
15 quality.

The replicable expression vectors may be prepared in accordance with the invention, by cleaving a vector compatible with the host cell to provide a linear DNA segment having an intact replicon, and combining said linear segment with one or more DNA molecules which, together with said linear segment encode the desired
20 product, such as the DNA polymer encoding the protein of the invention, or derivative thereof, under ligating conditions.

Thus, the DNA polymer may be preformed or formed during the construction of the vector, as desired.

The choice of vector will be determined in part by the host cell, which may be
25 prokaryotic or eukaryotic but preferably is *E. coli*. Suitable vectors include plasmids, bacteriophages, cosmids and recombinant viruses.

The preparation of the replicable expression vector may be carried out conventionally with appropriate enzymes for restriction, polymerisation and ligation of the DNA, by procedures described in, for example, Maniatis *et al.* cited above.

30 The recombinant host cell is prepared, in accordance with the invention, by transforming a host cell with a replicable expression vector of the invention under transforming conditions. Suitable transforming conditions are conventional and are

described in, for example, Maniatis *et al.* cited above, or "DNA Cloning" Vol. II, D.M. Glover ed., IRL Press Ltd, 1985.

The choice of transforming conditions is determined by the host cell. Thus, a bacterial host such as *E. coli* may be treated with a solution of CaCl_2 (Cohen *et al.*, Proc. Nat. Acad. Sci., 1973, 69, 2110) or with a solution comprising a mixture of RbCl, MnCl_2 , potassium acetate and glycerol, and then with 3-[N-morpholino]-propane-sulphonic acid, RbCl and glycerol. Mammalian cells in culture may be transformed by calcium co-precipitation of the vector DNA onto the cells. The invention also extends to a host cell transformed with a replicable expression vector of the invention.

Culturing the transformed host cell under conditions permitting expression of the DNA polymer is carried out conventionally, as described in, for example, Maniatis *et al.* and "DNA Cloning" cited above. Thus, preferably the cell is supplied with nutrient and cultured at a temperature below 50°C .

The product is recovered by conventional methods according to the host cell. Thus, where the host cell is bacterial, such as *E. coli* it may be lysed physically, chemically or enzymatically and the protein product isolated from the resulting lysate. Where the host cell is mammalian, the product may generally be isolated from the nutrient medium or from cell free extracts. Conventional protein isolation techniques include selective precipitation, adsorption chromatography, and affinity chromatography including a monoclonal antibody affinity column.

When the proteins of the present invention are expressed with a histidine tail (His tag). The proteins can easily be purified by affinity chromatography using an ion metal affinity chromatography column (IMAC) column.

A second chromatographic step, such as Q-sepharose may be utilised either before or after the IMAC column to yield highly purified protein. If the immunological fusion partner is C-LYTA, then it is possible to exploit the affinity of CLYTA for choline and/or DEAE to purify this product. Products containing both C-LYTA and his tags can be easily and efficiently purified in a two step process involving differential affinity chromatography. One step involves the affinity of the His tag to IMAC columns, the other involves the affinity of the C-terminal domain of LYTA for choline or DEAE.

A preferred vaccine composition comprises at least Protein D - E6 from HPV 16 or derivative thereof together with Protein D - E7 from HPV 16. Alternatively the E6 and E7 may be presented in a single molecule, preferably a Protein D E6/E7 fusion. Such vaccine may optionally contain either or both E6 and E7 proteins from HPV 18, preferably in the form of a Protein D - E6 or Protein D - E7 fusion protein or Protein D E6/E7 fusion protein. The vaccines of the present invention may contain other HPV antigens from HPV 16 or 18. In particular, the vaccine may contain L1 or L2 antigen monomers. Alternatively such L1 or L2 antigens may be presented together as a virus like particle or the L1 alone protein may be presented as virus like particle or capsomer structure. Such antigens, virus like particles and capsomer are per se known. See for example WO94/00152, WO94/20137, WO94/05792, and WO93/02184. Additional early proteins may be included such as E2 or preferably E5 for example. The vaccine of the present invention may additionally comprise antigens from other HPV strains, preferably from strains HPV 6, 11, 31 or 33.

Vaccine preparation is generally described in Vaccine Design - The subunit and adjuvant approach (Ed. Powell and Newman) Pharmaceutical Biotechnology Vol. 6 Plenum Press 1995. Encapsulation within liposomes is described by Fullerton, US Patent 4,235,877.

The preferred oligonucleotides preferably contain two or more CpG motifs separated by six or more nucleotides. The oligonucleotides of the present invention are typically deoxynucleotides. In a preferred embodiment the internucleotide in the oligonucleotide is phosphorodithioate, or more preferably a phosphorothioate bond, although phosphodiester and other internucleotide bonds are within the scope of the invention including oligonucleotides with mixed internucleotide linkages.

Preferred oligonucleotides have the following sequences: The sequences preferably contain all phosphorothioate modified internucleotide linkages.

OLIGO 1: TCC ATG ACG TTC CTG ACG TT

OLIGO 2: TCT CCC AGC GTG CGC CAT

OLIGO 3: ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG

The CpG oligonucleotides utilised in the present invention may be synthesized by any method known in the art (eg EP 468520). Conveniently, such oligonucleotides may be synthesized utilising an automated synthesizer. Methods for producing

phosphorothioate oligonucleotides or phosphorodithioate are described in US5,666,153, US5,278,302 and WO95/26204.

The invention will be further described by reference to the following examples:

5 **EXAMPLE I: Construction of an E. coli strain expressing fusion Protein-D1/3 - E7 -His (HPV16)**

1) - Construction of expression plasmid

a) - Plasmid pMG MCS prot D1/3 (= pRIT14589) is a derivative of pMG81 (described in UK patent application n° 951 3261.9 published as WO97/01640) in which the codons 4-81 of NS1 coding region from Influenza were replaced by the codons corresponding to residues Ser 20 → Thr127 of mature protein D of Haemophilus Influenzae strain 772, biotype 2 (H. Janson *et al.*, 1991, Infection and Immunity, Jan. p.119-125). The sequence of Prot-D1/3 is followed by a multiple cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His).
10 This plasmid is used to express the fusion protein D1/3-E7-His.

b) - HPV genomic E6 and E7 sequences type HPV 16 (See Dorf *et al.*, Virology 1985, 145, p. 181-185) were amplified from HPV 16 full length genome cloned in pBR322 (obtained from Deutsches Krebsforschungszentrum (DKFZ), Referenzzentrum für human pathogen Papillomaviruses - D 69120 - Heidelberg) and
20 were subcloned into pUC19 to give TCA 301 (= pRIT14462).

Construction of plasmid TCA 308 (= pRIT14501): a plasmid expressing the fusion Protein-D1/3-E7-His

The nucleotides sequences corresponding to amino acids 1 → 98 of E7 protein are amplified from pRIT14462. During the polymerase chain reaction, NcoI and SpeI restriction sites were generated at the 5' and 3' ends of the E7 sequences
25 allowing insertion into the same sites of plasmid pMG MCS Prot D1/3 to give plasmid TCA308 (= pRIT14501). The insert was sequenced to verify that no modification had been generated during the polymerase chain reaction. The sequence for the fusion protein-D1/3-E7-His (HPV 16) is described in sequence ID No.1 and the coding
30 sequence in ID No.2.

2) - Transformation of AR58 strain

Plasmid pRIT14501 was introduced into *E. coli* AR58 (Mott *et al.*, 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

3) - Growth and induction of bacterial strain - Expression of Prot -D1/3-E7-His

5 Cells of AR58 transformed with plasmid pRIT14501 were grown in 100 ml of LB medium supplemented with 50 μ g/ml of Kanamycin at 30°C. During the logarithmic phase of growth bacteria were shifted to 39°C to inactivate the λ repressor and turn on the synthesis of protein D1/3-E7-His. The incubation at 39°C was continued for 4 hours. Bacteria were pelleted and stored at -20°C.

10 EXAMPLE II: Construction of an *E. coli* strain expressing fusion Protein-D1/3-E6-his / HPV16

1. Construction of expression plasmid

a) Plasmid pMG MCS prot D1/3 (= pRIT14589) is a derivative of pMG81 (described in WO97/01640 in which the codons 4-81 of NS1 coding region from Influenza were
15 replaced by the codons corresponding to residues Ser 20 \rightarrow Thr 127 of mature protein D of Haemophilus Influenzae strain 772, biotype 2 (H. Janson *et al.*, 1991, Infection and Immunity, Jan. p.119-125). The sequence of Prot-D1/3 is followed by a multiple cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His). This plasmid is used to express the fusion protein D1/3-E6-his.

20 b) HPV genomic E6 and E7 sequences type HPV16 (Seedorf *et al.*, Virology 1985, 145, p.181-185) were amplified from HPV16 full length genome cloned in pBR322 (obtained from Deutsches Krebsforschungszentrum (DKFZ), Referenzzentrum für human pathogen Papillomaviruses -

c) D 69120 - Heidelberg) and were subcloned into pUC19 to give TCA 301 (= 25 pRIT14462).

Construction of plasmid TCA 307 (=pRIT14497) : a plasmid expressing the fusion Protein-D1/3-E6-His /HPV16

The nucleotides sequences corresponding to amino acid.

1 \rightarrow 151 of E6 protein were amplified from pRIT14462. During the
30 polymerase chain reaction, NcoI and SpeI restriction sites were generated at the 5' and 3' ends of the E6 sequences allowing insertion into the same sites of plasmid pMG MCS Prot D1/3 to give plasmid TCA307 (= pRIT14497). The insert was

sequenced to verify that no modification had been generated during the polymerase chain reaction. The protein and coding sequence for the fusion protein-D1/3-E6-His is described in sequence ID No.3 and 4.

2. Transformation of AR58 strain

5 Plasmid pRIT14497 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

3. Growth and induction of bacterial strain - Expression of Prot-D1/3-E6-His

Cells of AR58 transformed with plasmid pRIT14497 were grown in 100 ml of
10 LB medium supplemented with 50 μ g/ml of Kanamycin at 30°C. During the logarithmic phase of growth bacteria were shifted to 39°C to inactivate the λ repressor and turn on the synthesis of protein D1/3-E6-his. The incubation at 39°C was continued for 4 hours. Bacteria were pelleted and stored at -20°C.

4. Characterization of fusion Protein D1/3-E6-his (HPV 16)

15 Preparation of extracts

Frozen cells are thawed and resuspended in 10 ml of PBS buffer. Cells are broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract is centrifuged at 16.000 g for 30 minutes at 4°C.

Analysis on Coomassie-stained SDS-polyacrylamide gels and Western blots

20 After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

A major band of about 32 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal anti-protein-D and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase
25 (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 5 % of total protein.

5. Coexpression with thioredoxin

In an analogous fashion to the expression of prot D 1/3 E7 His from HPV 18
30 (example IX) an *E. coli* strain AR58 was transformed with a plasmid encoding thioredoxin and protein D 1/3 E7 His (HPV 16).

EXAMPLE III: Construction of an *E. coli* strain expressing fusion Protein-D1/3-E6E7-his / HPV16

1. Construction of expression plasmid

- a) Plasmid pMG MCS prot D1/3 (= pRIT14589) is a derivative of pMG81 (described
5 Supra) in which the codons 4-81 of NS1 coding region from Influenza were replaced
by the codons corresponding to residues Ser 20 → Thr 127 of mature protein D of
Haemophilus Influenzae strain 772, biotype 2 (H. Janson *et al.*, 1991, Infection and
Immunity, Jan. p.119-125). The sequence of Prot-D1/3 is followed by a multiple
cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His).
10 This plasmid is used to express the fusion protein D1/3-E6E7-his.
b) HPV genomic E6 and E7 sequences type HPV16 (Seedorf *et al.*, Virology 1985,
145, p.181-185) were amplified from HPV16 full length genome cloned in pBR322
(obtained from Deutsches Krebsforschungszentrum (DKFZ), Referenzzentrum für
human pathogen Papillomaviruses - D 69120 - Heidelberg) and were subcloned into
15 pUC19 to give TCA 301 (= pRIT14462).
c) The coding sequences for E6 and E7 in TCA301 (= pRIT
14462) were modified with a synthetic oligonucleotides adaptor (inserted between Afl
III and Nsi I sites) introducing a deletion of 5 nucleotides between E6 and E7 genes
to remove the stop codon of E6 and create fused E6 and E7 coding sequences in the
20 plasmid TCA309(= pRIT 14556).

**Construction of plasmid TCA 311(= pRIT14512) : a plasmid expressing the
fusion Protein-D1/3-E6E7-His /HPV16**

- The nucleotides sequences corresponding to amino acids 1 → 249 of fused
E6E7 protein were amplified from pRIT14556. During the polymerase chain
25 reaction, NcoI and SpeI restriction sites were generated at the 5' and 3' ends of the
E6E7 fused sequences allowing insertion into the same sites of plasmid pMGMCs
Prot D1/3 to give plasmid TCA311 (= pRIT14512). The insert was sequenced to
verify that no modification had been generated during the polymerase chain reaction.
The protein and coding sequence for the fusion protein-D E6/E7 1/3-His is described
30 sequence ID No. 5 and 6.

2. Transformation of AR58 strain

Plasmid pRIT14512 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

3. Growth and induction of bacterial strain - Expression of Prot-D1/3-E6E7-His

5 Cells of AR58 transformed with plasmid pRIT14512 were grown in 100 ml of LB medium supplemented with 50 μ g/ml of Kanamycin at 30°C. During the logarithmic phase of growth bacteria were shifted to 39°C to inactivate the λ repressor and turn on the synthesis of protein D1/3-E6E7-his. The incubation at 39°C was continued for 4 hours. Bacteria were pelleted and stored at -20°C.

10 4. Characterization of fusion Protein D1/3-E6E7-his

Frozen cells are thawed and resuspended in 10 ml of PBS buffer. Cells are broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract is centrifuged at 16.000 g for 30 minutes at 4°C.

15 After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

A major band of about 48 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal anti-protein-D and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase
20 (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 1 % of total protein.

EXAMPLE: IV

In an analogous fashion the fusion protein of Lipo D 1/3 and E6-E7 from HPV16 was expressed in *E. coli* in the presence of thioredoxin.

25 The N-terminal of the pre-protein (388 aa) contains MDP residues followed by 16 amino acids of signal peptide of lipoprotein D (from Haemophilus Influenzae) which is cleaved in vivo to give the mature protein (370 aa). Lipoprotein portion (aa 1 to 127) is followed by the proteins E6 and E7 in fusion. The C terminal of the protein is elongated by TSGHHHHHH.

30 EXAMPLE V: Construction of E.coli strain B1002 expressing fusion ProtD1/3-E7

Mutated (cys24->gly,glu26->gln) type HPV16

1)-Construction of expression plasmid**Starting material:**

- a) - Plasmid pRIT 14501 (= TCA 308) which codes for fusion ProtD1/3-E7 -His
- b) - Plasmid LITMUS 28 (New England Biolabs cat n° 306-28) , a cloning vector
- 5 pUC-derived
- c) - Plasmid pMG MCS ProtD1/3 (pRIT 14589) , a derivative of pMG81 (described Supra) in which the codons 4-81 of NS1 coding region from Influenza were replaced by the codons corresponding to residues Ser 20 → Thr 127 of mature protein D of Haemophilus Influenzae strain 772, biotype 2 (H. Janson *et al.*, 1991, Infection and
- 10 Immunity, Jan. p.119-125). The sequence of Prot-D1/3 is followed by a multiple cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His)

Construction of plasmid pRIT 14733(=TCA347): a plasmid expressing the fusion Protein-D1/3-E7 mutated (cys24->gly ,glu26->gln) with His tail

- The NcoI - XbaI fragment from pRIT 14501 (=TCA 308) , bearing the coding
- 15 sequence of E7 gene from HPV16 , elongated with an His tail , was subcloned in an intermediate vector Litmus 28 useful for mutagenesis to give pRIT 14909 (=TCA337)
- Double mutations cys24->gly (Edmonds and Vousden , J.Virology 63 : 2650 (1989) and glu26->gln (Phelps et al , J.Virology 66: 2418-27 (1992) were chosen to impair the binding to the antioncogene product of Retinoblastome gene (pRB).
- 20 The introduction of mutations in E7 gene was realized with the kit " Quick Change Site directed Mutagenesis (Stratagene cat n° 200518) to give plasmid pRIT 14681(=TCA343) .After verification of presence of mutations and integrity of the complete E7 gene by sequencing , the mutated E7 gene was introduced into vector pRIT 14589 (= pMG MCS ProtD1/3) to give plasmid pRIT 14733 (=TCA347)
- 25 protein and coding sequence.

The sequence for the fusion protein-D1/3-E 7 mutated (cys24->gly, glu26->gln) -His is described in sequence ID No. 7 and 8.

2)-Construction of strain B1002 expressing ProtD1/3-E7mutated (cys 24->gly , glu26->gln)-His /HPV16

- 30 Plasmid pRIT 14733 was introduced into *E.coli* AR58 (Mott et al. ,1985,

Proc. Natl. Acad. Sci. , 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter ,to give strain B1002 , by selection for transformants resistant to kanamycine

3)-Growth and induction of bacterial strain B1002 - Expression of ProtD1/3-E7 mutated (cys 24->gly , glu26->gln)-His /HPV16

Cells of AR58 transformed with plasmid pRIT 14733 (B1002 strain) were grown at 30°C in 100 ml of LB medium supplemented with 50 μ gr /ml of Kanamycin. During the logarithmic phase of growth bacteria were shifted to 39°C to inactivate the λ repressor and turn on the synthesis of ProtD1/3-E7 mutated -His /HPV16 . The incubation at 39°C was continued for 4 hours . Bacteria were pelleted and stored at -20°C.

4)-Characterization of fusion ProtD1/3-E7 mut (cys24->gly, glu26->gln)- His type HPV16.

Frozen cells were thawed and resuspended in 10 ml of PBS buffer. Cells were broken in a French Pressure cell press SLM Aminco at 20 000 psi (three passages) . The extract was centrifuged at 16000 g for 30 minutes at 4°C.

After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

A major band of about 33 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal 22 J 70 anti-protein D, by monoclonal anti E7 /HPV16 from Zymed and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 3 to 5 % of total protein.

Cells of B1002 were separated from the culture broth by centrifugation. The concentrated cells of B1002 were stored at -65°C.

EXAMPLE VI: Construction of an *E. coli* strain expressing fusion clyta-E6-his (HPV 16)

1. Construction of expression plasmid

a) -Plasmid pRIT14497 (= TCA307), that codes for fusion ProtD1/3-E6-His /HPV16

- b)-Plasmid pRIT14661 (= DVA2), an intermediate vector containing the coding sequence for the 117 C-terminal codons of LytA of *Streptococcus Pneumoniae*. LytA is derived from *Streptococcus pneumoniae* which synthesize an N-acetyl-L-alanine amidase, amidase LYTA. (coded by the *lytA* gene {Gene, 43 (1986) pag 265-272} an autolysin that specifically degrades certain bonds in the peptidoglycan backbone .
- 5 The C-terminal domain of the LYTA protein is responsible for the affinity to the choline or to some choline analogues such as DEAE.

1.b Construction of plasmid pRIT14634 (=TCA332): a plasmid expressing the fusion clyta-E6-His /HPV16

- 10 a)The first step was the purification of the large NcoI-AflIII restriction fragment from plasmid pRIT14497 and the purification of the small AflII-AflIII restriction fragment from pRIT14661
- b)The second step was linking of clyta sequences to the E7-His sequences (NcoI and AflIII are compatible restriction sites) that gave rise to the plasmid pRIT 14634
- 15 (=TCA332), coding for the fusion protein clyta-E6-His under the control of the pL promoter.

The protein and coding sequence for the fusion protein clyta-E6-His is described sequence ID No. 9 and 10.

Transformation of AR58 strain

- 20 Plasmid pRIT14634 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

Growth and induction of bacterial strain - Expression of clyta-E6-His

- Cells of AR58 transformed with plasmid pRIT14634 were grown in 100 ml of
- 25 LB medium supplemented with 50 μ gr/ml of Kanamycin at 30°C. During the logarithmic phase of growth bacteria were shifted to 39°C to inactivate the λ repressor and turn on the synthesis of protein clyta-E6-his. The incubation at 39°C was continued for 4 hours. Bacteria were pelleted and stored at -20°C.

4. Characterization of fusion clyta-E6-his

- 30 Frozen cells were thawed and resuspended in 10 ml of PBS buffer. Cells were broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract was centrifuged at 16.000 g for 30 minutes at 4°C. After centrifugation of

extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

A major band of about 33 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal anti-clyta antibodies and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 3 % of total protein.

EXAMPLE VII: Construction of an *E. coli* strain expressing fusion clyta-E7-his (HPV 16)

1. Construction of expression plasmid

1.a Starting materials

- a) -Plasmid pRIT14501 (= TCA308), that codes for fusion ProtD1/3-E7-His /HPV16
- b)-Plasmid pRIT14661 (= DVA2), an intermediate vector containing the coding sequence for the 117 C-terminal codons of LytA of *Streptococcus Pneumoniae*.

1.b Construction of plasmid pRIT14626 (=TCA330): a plasmid expressing the fusion clyta-E7-His / HPV16

- a) The first step was the purification of the large NcoI-AflIII restriction fragment from plasmid pRIT14501 and the purification of the small AflIII-AflIII restriction fragment from pRIT14661
- b) The second step was linking of clyta sequences to the E7-His sequences (NcoI and AflIII are compatible restriction sites) that gave rise to the plasmid pRIT 14626 (=TCA330), coding for the fusion protein clyta-E7-His under the control of the pL promoter.

The protein and coding sequence for the fusion protein clyta-E7-His is described in sequence ID No. 11 and 12.

2. Transformation of AR58 strain

Plasmid pRIT14626 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

3. Growth and induction of bacterial strain - Expression of clyta-E7-His

Cells of AR58 transformed with plasmid pRIT14626 were grown in 100 ml of LB medium supplemented with 50 μ gr/ml of Kanamycin at 30°C. During the

logarithmic phase of growth bacteria were shifted to 39°C to inactivate the λ repressor and turn on the synthesis of protein clyta-E7-his. The incubation at 39°C was continued for 4 hours. Bacteria were pelleted and stored at -20°C.

4. Characterization of fusion clyta-E7-his

5 Frozen cells were thawed and resuspended in 10 ml of PBS buffer. Cells were broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract was centrifuged at 16.000 g for 30 minutes at 4°C. After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

10 A major band of about 35 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal anti-clyta antibodies and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 5 % of total protein.

15 **EXAMPLE VIII: Construction of an *E. coli* strain expressing fusion clyta-E6E7-his (HPV 16)**

1. Construction of expression plasmid

1.a Starting materials

- a) -Plasmid pRIT14512 (= TCA311), that codes for fusion ProtD1/3-E6E7-His
20 /HPV16
- b)-Plasmid pRIT14661 (= DVA2), an intermediate vector containing the coding sequence for the 117 C-terminal codons of LytA of *Streptococcus Pneumoniae*.

1.b Construction of plasmid pRIT14629 (=TCA331): a plasmid expressing the fusion clyta-E6E7-His /HPV16

- 25 a)The first step was the purification of the large NcoI-AflIII restriction fragment from plasmid pRIT14512 and the purification of the small AflIII-AflIII restriction fragment from pRIT14661
- b)The second step was linking of clyta sequences to the E7-His sequences (NcoI and AflIII are compatible restriction sites)that gave rise to the plasmid pRIT 14629
30 (=TCA331), coding for the fusion protein clyta-E6E7-His under the control of the pL promoter.

The protein and coding sequence for the fusion protein clyta-E6E7-His is sequenced ID No. 13 and 14.

2. Transformation of AR58 strain

Plasmid pRIT14629 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

3. Growth and induction of bacterial strain - Expression of clyta-E6E7-His

Cells of AR58 transformed with plasmid pRIT14629 were grown in 100 ml of LB medium supplemented with 50 μ g/ml of Kanamycin at 30°C. During the logarithmic phase of growth bacteria were shifted to 39°C to inactivate the λ repressor and turn on the synthesis of protein clyta-E6E7-his. The incubation at 39°C was continued for 4 hours. Bacteria were pelleted and stored at -20°C.

4. Characterization of fusion clyta-E6E7-his

Frozen cells were thawed and resuspended in 10 ml of PBS buffer. Cells were broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract was centrifuged at 16.000 g for 30 minutes at 4°C.

After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

A major band of about 48 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal anti-clyta antibodies and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 1 % of total protein.

25 EXAMPLE IX: Prot D1/3 E7 his (HPV 18) (E.Coli B1011)

Protein D1/3 E7 his HPV expressed with Thioredoxin inTrans (E.Coli B1012)

1) - Construction of expression plasmids

1).a.Construction of plasmid TCA316(=pRIT 14532) a plasmid expressing the fusion Protein-D1/3-E7-His /HPV18

30 Starting materials

a) - Plasmid pMG MCS prot D1/3 (= pRIT14589) is a derivative of pMG81 (described in UK patent application n° 951 3261.9 published as WO97/01640 in

which the codons 4-81 of NS1 coding region from Influenza were replaced by the codons corresponding to residues Ser 20 → Thr 127 of mature protein D of Haemophilus Influenzae strain 772, biotype 2 (H. Janson *et al.*, 1991, Infection and Immunity, Jan. p.119-125). The sequence of Prot-D1/3 is followed by a multiple cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His). This plasmid is used to express the fusion protein D1/3-E7-his.

b) - HPV genomic E6 and E7 sequences of prototype HPV18(Cole et al.J.Mol.Biol.(1987)193,599-608) were amplified from HPV16 full length genome cloned in pBR322 (obtained from Deutsche Krebsforschungszentrum (DKFZ), Referenzzentrum für human pathogen Papillomaviruses - D 69120 - Heidelberg) and were subcloned into pUC19 to give TCA 302 (= pRIT14467).

Construction of plasmid TCA 316(= pRIT14532)

The nucleotides sequences corresponding to amino acids 1 → 105 of E7 protein were amplified from pRIT14467. During the polymerase chain reaction, NcoI and SpeI restriction sites were generated at the 5' and 3' ends of the E7 sequences allowing insertion into the same sites of plasmid pMGMCs Prot D1/3 to give plasmid TCA316 (= pRIT14532). The insert was sequenced and a modification versus E7/HPV18 prototype sequence was identified in E7 gene (nucleotide 128 G->A) generating a substitution of a glycine by a glutamic acid (aa 43 in E7 , position 156 in fusion protein). The protein and coding sequence for the fusion protein-D1/3-E7-His /HPV18 is set forth in sequence ID No. 15 and ID No. 16.

1).b. Construction of plasmid TCA313 (=pRIT14523): a plasmid expressing thioredoxin

Starting materials

- a) - Plasmid pBBR1MCS4(Antoine R. and C.Locht,Mol.Microbiol. 1992,6,1785-1799 ; M.E.Kovach et al. Biotechniques 16, (5), 800-802)which is compatible with plasmids containing ColE1 or P15a origins of replication.
- b) - Plasmid pMG42 (described in WO93/04175) containing the sequence of promoter pL of Lambda phage
- c) - Plasmid pTRX (Invitrogen, kit Thiofusion K350-01) bearing the coding sequence for thioredoxin followed by AspA transcription terminator.

Construction of plasmid TCA313(=pRIT14523)

The fragment EcoRI-NdeI fragment from pMG42, bearing pL promoter and the NdeI-HindIII fragment from pTRX, bearing the coding sequence for thioredoxin followed by AspA terminator, were purified and ligated into the EcoRI and HindIII sites of plasmid vector pBBR1MCS4 to give plasmid TCA313(= pRIT14523).

5 The coding sequence for thioredoxin is described in ID No. 17.

2) - Transformation of AR58 strain

2).a. To obtain strain B1011 expressing ProtD1/3-E7-His/HPV18

Plasmid pRIT14532 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the
10 λ pL promoter, by selection for transformants resistant to kanamycine.

2).b. Construction of strain B1012 expressing ProtD1/3-E7-His/HPV18 and thioredoxin

Plasmid pRIT14532 and pRIT14523 were introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a
15 thermosensitive repressor of the λ pL promoter, by double selection for transformants resistant to kanamycin and ampicillin.

3) - Growth and induction of bacterial strains B1011 and B1012 - Expression of Prot-D1/3-E7-His/HPV18 without and with thioredoxin in trans

Cells of AR58 transformed with plasmids pRIT14532 (B1011 strain) and
20 Cells of AR58 transformed with plasmids pRIT14532 and pRIT14523 (B1012 strain) were grown at 30°C in 100 ml of LB medium supplemented with 50 μ gr/ml of Kanamycin for B1011 strain and supplemented 50 μ gr/ml of Kanamycin and 100 μ gr/ml of Ampicillin for B1012 strain. During the logarithmic phase of growth bacteria were shifted to 39°C to inactivate the λ repressor and turn on the synthesis
25 of protein D1/3-E7-his/HPV18 and thioredoxin. The incubation at 39°C was continued for 4 hours.

Characterization of fusion Protein D1/3-E7-his /HPV18

Preparation of extracts

Frozen cells are thawed and resuspended in 10 ml of PBS buffer. Cells are
30 broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract is centrifuged at 16.000 g for 30 minutes at 4°C.

Analysis on Coomassie-stained SDS-polyacrylamide gels and Western blots

After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

The fusion protD1/3-E7-His (about 31 kDa) was visualised by Coomassie stained gels in the pellet fraction for strain B1011 and partially localized (30%) in the supernatant fraction for strain B1012 and was identified in Western blots by rabbit polyclonal anti-protein-D and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 1-3% of total protein as shown on a Coomassie-stained SDS-polyacrylamide gel.

For the extract of strain B1012 the thioredoxin (about 12 KDa) was visualised by coomassie stained gel in the supernatant and identified in western blots by monoclonal anti thioredoxin (Invitrogen R920-25)

EXAMPLE X: Construction of E.coli strain B1098 expressing fusion ProtD1/3-E7

Mutated (cys27->gly,glu29->gln) type HPV18

1)-Construction of expression plasmid

Starting material:

- a) - Plasmid pRIT 14532 (= TCA 316) which codes for fusion ProtD1/3-E7 -His
- 20 b) - Plasmid LITMUS 28 (New England Biolabs cat n° 306-28) , a cloning vector pUC-derived
- c) - Plasmid pMG MCS ProtD1/3 (pRIT 14589) , a derivative of pMG81 (described supra) in which the codons 4-81 of NS1 coding region from Influenza were replaced by the codons corresponding to residues Ser 20 → Thr 127 of mature protein D of
- 25 Haemophilus Influenzae strain 772, biotype 2 (H. Janson *et al.*, 1991, Infection and Immunity, Jan. p.119-125). The sequence of Prot-D1/3 is followed by a multiple cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His)

Construction of plasmid pRIT 14831 (=TCA355): a plasmid expressing the fusion Protein-D1/3-E7 mutated (cys27->gly ,glu29->gln) with His tail

- 30 The NcoI - XbaI fragment from pRIT 14532 (=TCA 316) , bearing the coding sequence of E7 gene from HPV18 , elongated with an His tail , was subcloned in an intermediate vector Litmus 28 useful for mutagenesis to give pRIT 14910 (=TCA348)

By analogy with E7/HPV16 mutagenesis, double mutations cys27-->gly and glu29-->gln were chosen to impair the binding to the antioncogene product of Retinoblastome gene (pRB).

The introduction of mutations in E7 gene was realized with the kit " Quick Change Site directed Mutagenesis (Stratagene cat n° 200518) .As the sequencing of pRIT14532 had pointed out the presence of a glutamic acid in position 43 of E7 instead of a glycine in the prototype sequence of HPV18 , a second cycle of mutagenesis was realized to introduce a glycine in position 43 . We obtained plasmid pRIT 14829 (= TCA353). After verification of presence of mutations and integrity of the complete E7 gene by sequencing , the mutated E7 gene was introduced into vector pRIT 14589 (= pMG MCS ProtD1/3) to give plasmid pRIT 14831 (=TCA355).

The protein and coding sequence for the fusion protein-D1/3-E 7 mutated (cys27->gly, glu29->gln) -His is described in sequence ID No. 18 and 19.

2)Construction of strain B1098 expressing ProtD1/3-E7mutated (cys 27-->gly , glu29-->gln)-His /HPV18

Plasmid pRIT 14831 was introduced into *E.coli* AR58 (Mott et al. ,1985, Proc. Natl. Acad. Sci. , 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter ,to give strain B1098 , by selection for transformants resistant to kanamycin.

3)-Growth and induction of bacterial strain B1098 - Expression of ProtD1/3-E7 mutated (cys 27->gly , glu29->gln)-His /HPV18

Cells of AR58 transformed with plasmid pRIT 14831 (B1098 strain) were grown at 30°C in 100 ml of LB medium supplemented with 50 μ gr /ml of Kanamycin. During the logarithmic phase of growth bacteria were shifted to 39°C to inactivate the λ repressor and turn on the synthesis of ProtD1/3-E7 mutated -His /HPV18 . The incubation at 39°C was continued for 4 hours. Bacteria were pelleted and stored at - 20°C.

4)-Characterization of fusion ProtD1/3-E7 mut (cys24->gly, glu26->gln)- His type HPV16

Frozen cells were thawed and resuspended in 10 ml of PBS buffer. Cells were broken in a French Pressure cell press SLM Aminco at 20 000 psi (three passages) . The extract was centrifuged at 16000 g for 30 minutes at 4°C.

Analysis on Coomassie stained SDS-polyacrylamide gels and Western blots

- 5 After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting. A major band of about 31 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal 22 J 70 anti-protein D and by monoclonal Penta-His (Qiagen cat. n° 34660) which detects
10 accessible histidine tail. The level of expression represents about 3 to 5 % of total protein.

EXAMPLE XI: Construction of an *E. coli* strain expressing fusion Protein-D1/3-E6-his / HPV18

1. Construction of expression plasmid

- 15 a) Plasmid pMG MCS prot D1/3 (= pRIT14589) is a derivative of pMG81 (described supra) in which the codons 4-81 of NS1 coding region from Influenza were replaced by the codons corresponding to residues Ser 20 → Thr 127 of mature protein D of Haemophilus Influenzae strain 772, biotype 2 (H. Janson *et al.*, 1991, Infection and Immunity, Jan. p.119-125). The sequence of Prot-D1/3 is followed by a multiple
20 cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His). This plasmid is used to express the fusion protein D1/3-E6-his.
HPV genomic E6 and E7 sequences type HPV18 (Cole et al., J. Mol. Biol. 1987, 193 , p.599-608.) were amplified from HPV18 full length genome cloned in pBR322 (obtained from Deutsches Krebsforschungszentrum (DKFZ), Referenzzentrum für
25 human pathogen Papillomaviruses - D 69120 - Heidelberg) and were subcloned into pUC19 to give TCA 302 (= pRIT14467).

Construction of plasmid TCA 314(= pRIT14526) : a plasmid expressing the fusion Protein-D1/3-E6-His /HPV18

The nucleotides sequences corresponding to amino acids

- 30 1 → 158 of E6 protein were amplified from pRIT14467. During the polymerase chain reaction, NcoI and SpeI restriction sites were generated at the 5' and 3' ends of the E6 sequences allowing insertion into the same sites of plasmid

pMG MCS Prot D1/3 to give plasmid TCA314 (= pRIT14526). The insert was sequenced to verify that no modification had been generated during the polymerase chain reaction. The protein and coding sequence for the fusion protein-D1/3-E6-His is described in sequence ID No. 20 and 21.

5 Transformation of AR58 strain

Plasmid pRIT14526 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

3. Growth and induction of bacterial strain - Expression of Prot-D1/3-E6-His

10 Cells of AR58 transformed with plasmid pRIT14526 were grown in 100 ml of LB medium supplemented with 50 μ g/ml of Kanamycin at 30°C. During the logarithmic phase of growth bacteria were shifted to 39°C to inactivate the λ repressor and turn on the synthesis of protein D1/3-E6-his. The incubation at 39°C was continued for 4 hours. Bacteria were pelleted and stored at -20°C.

15 4. Characterization of fusion Protein D1/3-E6-his

Frozen cells are thawed and resuspended in 10 ml of PBS buffer. Cells are broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract is centrifuged at 16.000 g for 30 minutes at 4°C. After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-
20 polyacrylamide gel electrophoresis and Western blotting.

A major band of about 32 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal anti-protein-D and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression
25 represents about 3-5 % of total protein.

EXAMPLE XII: Construction of an *E. coli* strain expressing fusion Protein-D1/3-E6E7-his / HPV18

I. Construction of expression plasmid

a) Plasmid pMG MCS prot D1/3 (= pRIT14589) is a derivative of pMG81 (described
30 supra) in which the codons 4-81 of NS1 coding region from Influenza were replaced by the codons corresponding to residues Ser 20 \rightarrow Thr 127 of mature protein D of Haemophilus Influenzae strain 772, biotype 2 (H. Janson *et al.*, 1991, Infection and

Immunity, Jan. p.119-125). The sequence of Prot-D1/3 is followed by a multiple cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His). This plasmid is used to express the fusion protein D1/3-E6E7-his.

b) HPV genomic E6 and E7 sequences type HPV18 (Cole et al., J.Mol.Biol. 1987, 5 193, 599-608) were amplified from HPV18 full length genome cloned in pBR322 (obtained from Deutsches Krebsforschungszentrum (DKFZ), Referenzzentrum für human pathogen Papillomaviruses - D 69120 - Heidelberg) and were subcloned into pUC19 to give TCA 302 (= pRIT14467).

c) The coding sequences for E6 and E7 in TCA302 (= pRIT 10 14467) were modified with a synthetic oligonucleotides adaptor (inserted between Hga I and Nsi I sites) introducing a deletion of 11 nucleotides between E6 and E7 genes, removing the stop codon of E6 and creating fused E6 and E7 coding sequences in the plasmid TCA320(= pRIT 14618).

Construction of plasmid TCA 328(= pRIT14567) : a plasmid expressing the
15 **fusion Protein-D1/3-E6E7-His /HPV18**

The nucleotides sequences corresponding to amino acids

1 → 263 of fused E6E7 protein were amplified from pRIT14618. During the polymerase chain reaction, NcoI and SpeI restriction sites were generated at the 5' and 3' ends of the E6E7 fused sequences allowing insertion into the same sites of plasmid 20 pMG MCS Prot D1/3 to give plasmid TCA328 (= pRIT14567). The insert was sequenced to verify that no modification had been generated during the polymerase chain reaction. The protein and coding sequence for the fusion protein-D1/3-E6E7-His is described in sequence ID No. 22 and 23.

2. Transformation of AR58 strain

25 Plasmid pRIT14567 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

3. Growth and induction of bacterial strain - Expression of Prot-D1/3-E6E7-His

Cells of AR58 transformed with plasmid pRIT14512 were grown in 100 ml of 30 LB medium supplemented with 50 µg/ml of Kanamycin at 30°C. During the logarithmic phase of growth bacteria were shifted to 39°C to inactivate the λ repressor

and turn on the synthesis of protein D1/3-E6E7-his. The incubation at 39°C was continued for 4 hours. Bacteria were pelleted and stored at -20°C.

4.Characterization of fusion Protein D1/3-E6E7-his

Frozen cells are thawed and resuspended in 10 ml of PBS buffer. Cells are
5 broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages).
The extract is centrifuged at 16.000 g for 30 minutes at 4°C.

After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

10 A major band of about 48 kDa, localized in the pellet fraction, was visualised by
Coomassie stained gels and identified in Western blots by rabbit polyclonal anti-
protein-D and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase
(Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression
represents about 1 % of total protein.

15 EXAMPLE XIII

The therapeutic potential of vaccine containing the PD1/3 E7 fusion protein and different CpG oligonucleotides were evaluated in the TC1 (E7 expressing tumour model.)

1. Therapeutic experiments: protocol

20 10e6 TC1 cells, E7 expressing tumour cells: were injected subcutaneously
(200µl) in the flank of C57BL/6 immunocompetent mice. Mice were vaccinated 7
and 14 days after the tumour challenge, with 5µg ProtD 1/3 E7 HPV16 injected intra-
footpad (100µl : 50µl / footpad) in the presence of different adjuvants:

2 and 4 weeks after the second immunisation, 5 mice/group were killed and
25 spleens or popliteal lymph nodes were taken and analyzed for immune response.

1.2 Results

Groups of mice

- 1) PBS³
- 2) ProtD1/3 E7 HPV16
- 30 3) ProtD1/3 E7 HPV16 + oligo 1: 1826 (WD 1001): TCC ATG ACG TTC CTG ACG TT
- 4) Oligo 1
- 5) ProtD1/3 E7 HPV16 + oligo 2/ 1758 (WD1002): TCT CCC AGC GTG CGC CAT

6) Oligo 2

Tumour Growth;

was monitored by measuring individual tumours twice a week.

Figure 1 : represents the mean tumour growth (in mm²)/group n=10 followed
5 over 4 weeks.

- The injection of 10e6 TC1 cells injected subcutaneously give rise to a growing tumour in 100% of the animals.
- Vaccinating with ProtD1/3E7 or adjuvant alone: 100% of the animals develop a tumour.
- 10 • As shown in figure 1 and 2, in the groups of mice that received the antigen with a CpG oligonucleotide the mean tumour growth remained very low and very similar between groups, reflecting that the tumour growth either was slowed down or that several tumours were completely rejected.

The analysis of individual tumour growth 2 and 4 weeks after the latest
15 vaccination showed that complete rejection in the groups were:

	Day 28 (n=10)	day 42 (n=5)
E7+oligo1 (1826)	40%	40%
Oligo1	0%	0%
E7+oligo2 (1758)	70%	40%
Oligo2	0%	0%

The mean tumour growth/group of mice vaccinated with PD1/3 E7+ the CpG
oligos are quite similar and analysis of the individual tumour growth showed that the
20 CpG oligos induce prolonged complete tumour rejection.

Conclusion

Both CpG (Oligo 2>oligo 1) induced complete tumour regression .

Lymphoproliferative response was analysed by *in vitro* restimulation of
spleen and lymph nodes cells for 72 hrs with either PD1/3E7, the protein E7(Bollen)
25 and PD (whole) PD1/3 (coated or not on latex µbeads) (10, 1, 0.1 µg/ml) 2 and 4
weeks post II.

- Positive controls (ConA stimulation) were positive.

- Surprisingly, no E7 specific and no PD specific proliferative response could be observed starting with spleen cells 2 or 4 weeks post II (probably due to a technical problem: data not shown).
- On the contrary, lymph node cells from mice that received ProtD1/3 E7 in CpG oligos 1 and 2 showed a very good E7 specific proliferative response although almost no PD (whole) specific response could be observed even at the highest concentration of 100µg/ml no PD1/3 specific responses was observed even when coated on latex µbeads.

Similar data were obtained 4 weeks post II.

10 Serology

The anti E7 antibody response: IgG tot and isotypes (IgG1, IgG2a, IgG2b, IgGTot) were measured by ELISA using the E7 protein as coating antigen as described in the Materials and Methods. Figures 3 and 4 show the relative percentage of the different IgG isotypes in the total of IgGs, 2 and 4 weeks post II respectively.

- The Oligos affect only weakly (oligo 2) or not at all (Oligo 1) the weak antibody response observed when PD1/3E7 alone was injected.
- The predominant E7 specific antibody subclass was clearly IgG2b for all the formulation tested (80-90% of the total IgGs).

The same results were obtained 4 weeks post II

20 Isotypic profile of anti E7 responses (post II, pooled sera) exp. 97293

Groups	IgG1	IgG2a	IgG2b	IgGtot
1) PBS	0	0	0	0
2) ProtD1/3 E7 HPV16	1020	0	4130	4740
3) ProtD1/3 E7 HPV16 + oligo 1	170	400	3680	4910
4) Oligo 1	0	0	530	420
5) ProtD1/3 E7 HPV16 + oligo 2	0	590	7560	13690
6) Oligo 2	0	0	0	0

Groups	IgG1	IgG2a	IgG2b	IgGtot
1) PBS	0	0	0	0
2) ProtD1/3 E7 HPV1	240	0	1650	1400
3) ProtD1/3 E7 HPV16 + oligo 1	0	0	1280	1430
4) Oligo 1	0	0	0	0
5) ProtD1/3 E7 HPV16 + oligo 2	0	560	3600	5880
6) Oligo 2	0	0	0	0

CTL assay:

- A CTL response could be detected when measured 2 weeks after the latest vaccination, when cells were re-stimulated in vitro with irradiated TC1 when TC1 or peptide E7 pulsed EL4, were used as target cells, when mice immunised with PD1/3 E7 + CpG oligo 2> 1 (25-40% specific lysis) and not with oligos alone.
- Lysis was seen on TC1 cells than on peptide E7 pulsed EL4 cells, but this is mostly observed in the groups of mice vaccinated with PD1/3E7 + CpG oligos (2>1). In this experiment other formulations did not induce a CTL.
 - Using E7 pulsed EL4 cells, no lysis was observed when mice received the protein or the adjuvant alone.

1.3 Materials and Methods

Component	Brand	Batch number	Concentration (mg/ml)	Buffer
ProtD1/3-E7		957/015	0.677	PBS 7.4
oligo CpG 1826	EuroGentec	WD1001	5	H ₂ O
oligo CpG	EuroGentec	WD1002	5	H ₂ O

1.3.1 Formulation Process

- All the formulations were prepared on the day of injection.

Oligo containing formulations

Formulations containing oligo alone without other adjuvant were prepared by addition of CpG to the diluted PrtD1/3-E7 in PBS pH 7.4.

- The adjuvant controls without antigen were prepared by replacing the protein by PBS.

1.3.2 Mice and Cell lines

Mice C57Bl/6 (Iffa Credo) 6-8 weeks old mice were used in these experiments.

- Cell lines:** TC1 (obtained from the John Hopkin's University) , or EL4 cells were grown in RPMI 1640 (Bio Whittaker) containig 10% FCS and additives: 2mM L-Glutamine , 1% antibiotics (10000U/ml penicilin. 10000µg/ml streptomycin) 1% non essential amino acid 100x, 1% sodium pyruvate (Gibco), 5 10e-5 M 2-

mercaptoethanol. Before injection TC1 cells were trypsinized and washed in serum free medium.

1.3.3 Tumour growth:

All the animals were injected with tumor cells on day 0 and were randomized at day 7. Individual tumor growth was followed over time (the 2 main diameters (A, B) were measured using calipers twice a week, A x B represents the "tumor surface" and the average of the 5 values / groups is showed on a graphic over time: 6 weeks

1.3.4 CMI read out

In vitro lymphoproliferation

Lymphoproliferation was performed on individual spleens and on lymph node pools. 200000 spleen cells or popliteal lymph node cells were plated in triplicate, in 96 well microplate, in RPMI medium containing 1% normal mice serum and additives . After 72 hrs of in vitro re-stimulation with different amounts of PD1/3 E7 (1, 0.1, 0.01 $\mu\text{g/ml}$) or E7 (10-1-0.1 $\mu\text{g/ml}$) After 72hrs, 100 μl of culture supernatant were removed and replaced by fresh medium containing 1 μCi ^3H thymidine (Amersham 5Ci/mmol) . After 16 hrs, cells were harvested onto filter plates. Incorporated radioactivity was counted in a β counter. Results are expressed in CPM (mean of triplicate wells) or as stimulation indexes (mean CPM in cultures with antigen / mean CPM in cultures without antigen).

20

1.3.5 CTL assay

20 10e6 spleen cells were co-cultured with 2 10e6 irradiated (18000r) TC1 cells (E7 expressing tumor) for 7 days in the presenced or absence of ConA sup. (2%)

25 Target cells used to assess cytotoxicity were either Cr51 (DuPont NEN 37MBq/ml) loaded (1hr at 37°C) TC1 cells or E7 pulsed EL4 cells (for 1 hr at 37°C during the Cr 51 loading of the cells 10 $\mu\text{g/ml}$ of E7-derived peptide (49-57) (QCB) compared to EL4 cells NK dependant lysis was assessed on K562 target cells 2000 target cells were added / well of 96 well plate (V bottom nunc 2-45128) with 100/1 being the highest Effector / target ratio. Controls for spontaneous or maximal Cr51 release were performed in sextuplet and were targets in medium or in triton 1.5%. All plates were gently centrifuged and incubated for 4 hrs at 37 in 7% CO₂. 50 μl of the

30

supernatant was deposited on 96w Lumaplate (Packard) let dry O/N and counted in a Top Count counter. Data is expressed as percent specific lysis which is calculated from the c.p.m. by the formula (experimental release - spontaneous release) / (maximal release - spontaneous release) X 100.

5 **Serology**

Quantitation of anti E7 antibody was performed by Elisa using E7as coating antigen. Antigen and antibody solutions were used at 50 µl per well. Antigen was diluted at a final concentration of 3 µg/ml in carbonate buffer ph9.5 and was adsorbed overnight at 4°C to the wells of 96 wells microtiter plates (Maxisorb Immuno-plate, 10 Nunc, Denmark). The plates were then incubated for 1hr at 37°C with PBS containing 1% bovine serum albumin and 0.1% Tween 20 (saturation buffer). Two-fold dilutions of sera (starting at 1/100 dilution) in the saturation buffer were added to the E7-coated plates and incubated for 1 hr 30 min at 37°C. The plates were washed 3 times with PBS 0.1% Tween 20 and biotin-conjugated anti-mouse IgG1, IgG2a or IgG2b or 15 IgGtot (Amersham, UK) diluted 1/5000 in saturation buffer was added to each well and incubated for 1 hr 30 min at 37°C. After a washing step, streptavidin-biotinylated peroxylase complex (Amersham, UK) diluted 1/5000 in saturation buffer was added for an additional 30 min at 37°C. Plates were washed as above and incubated for 10 min with TMB(tetra-methyl-benzidine). The reaction was stopped with H2SO4 4N 20 and read at 450 nm. Midpoint dilutions were calculated by SoftmaxPro (using a four parameters equation).

EXAMPLE XIV

In a second experiment, the vaccine of the invention were tested to assess the significance of the backbone:

25 **Therapeutic experiment: protocol**

- 10e6 TC1 cells , E7 expressing tumor cells : were injected subcutaneously (200µl) in the flank of immunocompetent C57BL/6 mice.
- 30 • 2 vaccinations, 7 and 14 days after the tumor challenge, with 5µg ProtD 1/3 E7 HPV16 injected intra- footpad (100 µl : 50µl / footpad) +/- CpG oligo; Oligo 1 (WD1001) as a phosphorothioate modified or the same Oligo (WD1006) but with phosphodiester linkage.

- 5 animals /group.

The **tumor growth** was monitored by measuring individual tumors twice a week and the mean tumor growth/ group of 5 animals is depicted in figure 5 and show the phosphorothioate modified oligonucleotides are effective in bringing about tumour regression.

Conclusions:

10

- All the animals that received 10e6 TC1 tumor cells develop a growing tumor.
- 100% of the animals vaccinated twice, 7 days apart, with the PD1/3 E7 HPV16 protein alone develop a tumor.
- 15 • 100% of the animals receiving the PD1/3 E7 protein + oligo WD1006 develop a tumor at the concentrations tested
- All the groups of animals that received the E7 protein + CpG 1001 at a concentration ranging from 10 to 200µg show tumor regression partial or
- 20 complete(20-40%).

The first concentration at which this therapeutic effect on tumor regression is not fully obtained is E7+ 1µg CpG oligo 1001.

25 EXAMPLE XV

In a third series of experiments, the vaccines of the invention were evaluated in transgenic mice expressing E7 protein.

- 30 • The transgenic mouse strain has been generated by M. Parmentier and C. Ledent at the IRIBHN (ULB). (Ref: PNAS (USA) 1990, 87; 6176-6180).
- As transgenic mice live with the E7 HPV16 gene from birth, they are considered "tolerant" to this gene: E7 from HPV 16, in this situation is considered as a "self
- 35 antigen".

- The expression of the transgene is driven by the thyroglobulin promoter. As Thyroglobulin is constitutively expressed only In the Thyroid, E7 is expressed in the thyroid.
- 5 • As a consequence of this expression, thyroid cells proliferate, mouse develop goiter and nodules which after 6 months to 1 year can evolve in invasive cancer.

The results (figure 6) of the experiments show that therapeutic vaccination with CpG oligonucleotide and antigen as described herein, results in a reduction of tumour
10 growth and can induce complete tumour regression.

Material & Methods

- 10e6 TC1 cells, E7 expressing tumor cells : were injected subcutaneously
15 (200µl) in the flank of male or female C57BL/6 Transgenic
 - mice were vaccinated 7 and 14 days after the tumor challenge, with 5µg ProtD 1/3 E7 HPV16 injected intra- footpad (100 µl : 50µl / footpad) in the 2 presence of CpG oligonucleotide TCT CCC AGC GTG CGC CAT and two control
20 adjuvants;,
 - 10 animals /group
- 2 and 4 weeks after the second immunization were killed and spleens or popliteal
25 lymph.

Conclusion

The vaccines of the invention are effective in bringing about tumour regression in
30 HPV induced tumours.

CLAIMS

1. A composition comprising an E6 or E7 protein or E6/E7 fusion protein from HPV optionally linked to an immunological fusion partner, and an immunomodulatory
5 CpG oligonucleotide.
2. A composition as claimed in claim 1 wherein the fusion partner is selected from the group; protein D or a fragment thereof from *Haemophilus influenzae* B, lipoprotein D or fragment thereof from *Haemophilus influenzae* B, NS1 or fragment thereof from Influenzae Virus, and LYTA or fragment thereof from
10 *Streptococcus Pneumoniae*.
3. A composition as claimed in claim 1 or 2 wherein the E6 or E7 proteins are derived from HPV16 or HPV18.
4. A composition as claimed in claim 1, 2 or 3 wherein the E7 protein is mutated.
5. A composition as claimed in claim 1, 2 or 3 wherein the E6 protein is mutated.
- 15 6. A composition as claimed in any of claims 1 to 5 additionally comprising a histidine tag of at least 4 histidine residues.
7. A composition as claimed herein comprising an additional HPV antigen.
8. A composition as claimed herein where the immunomodulatory CpG oligonucleotide comprises a hexamer motif: purine purine cytosine guanine pyrimidine pyrimidine.
- 20 9. A composition as claimed herein wherein the immunomodulatory CpG oligonucleotide has two or more CpG motifs.
10. A composition as claimed herein wherein the CpG oligonucleotide contains a phosphorothioate inter-nucleotide linkage.
11. A composition as claimed herein wherein the CpG oligonucleotide is selected
25 from the group:

OLIGO 1: TCC ATG ACG TTC CTG ACG TT

OLIGO 2: TCT CCC AGC GTG CGC CAT

OLIGO 3: ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG

12. A composition as claimed herein for use in medicine.
- 5 13. A method of inducing an immune response in a patient to an HPV antigen comprising administering a safe and effective amount of a composition as claimed herein.
14. A method of preventing or treating HPV induced tumours in a patient comprising administering a safe and effective amount of a composition as claimed herein.
- 10 15. A method of preparing a composition as claimed herein, comprising admixing an E6, E7 or E6/E7 fusion protein optionally linked to an immunological fusion partner, and an immunomodulatory CpG oligonucleotide.

Fig. 1

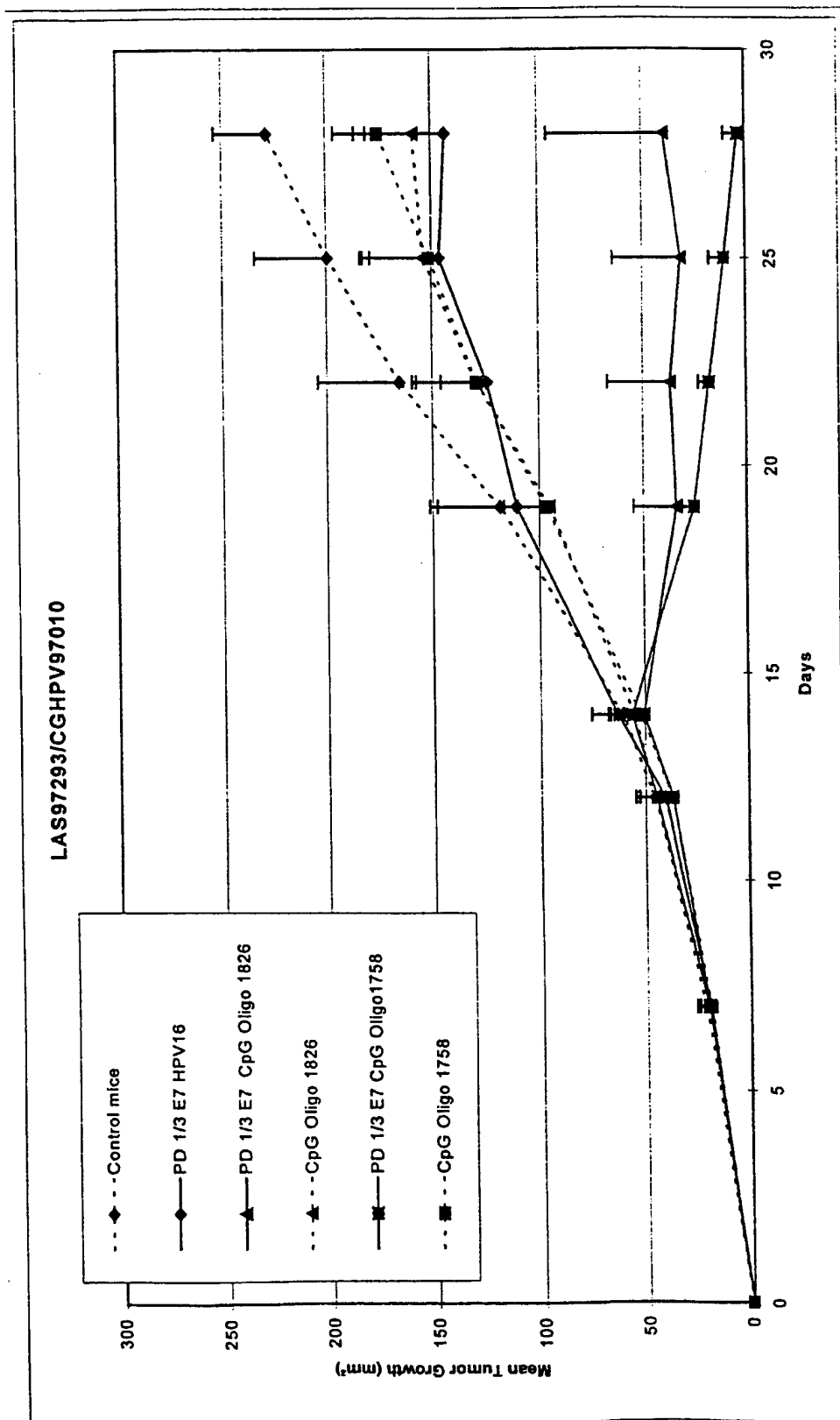


Fig. 2

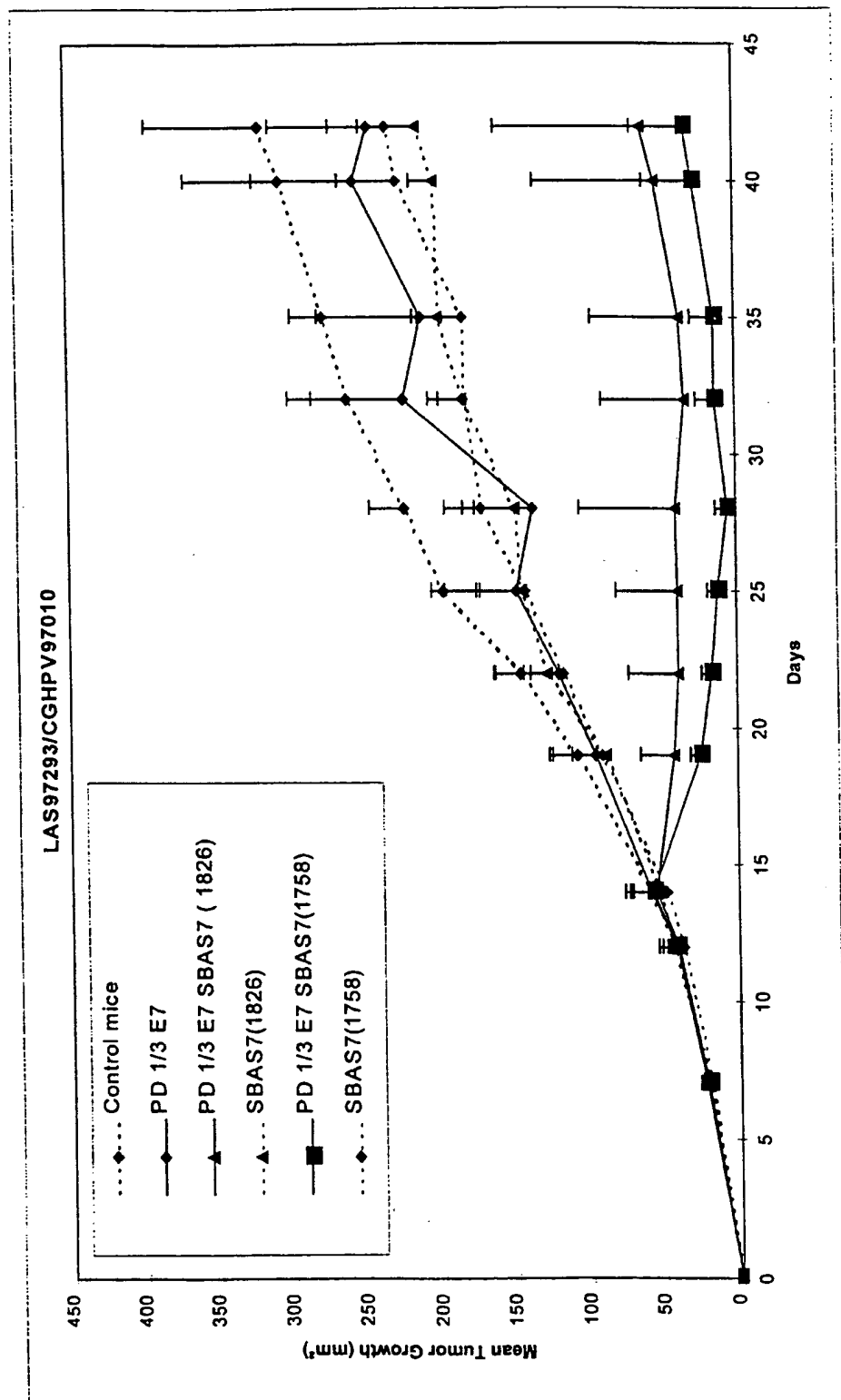


Fig. 3

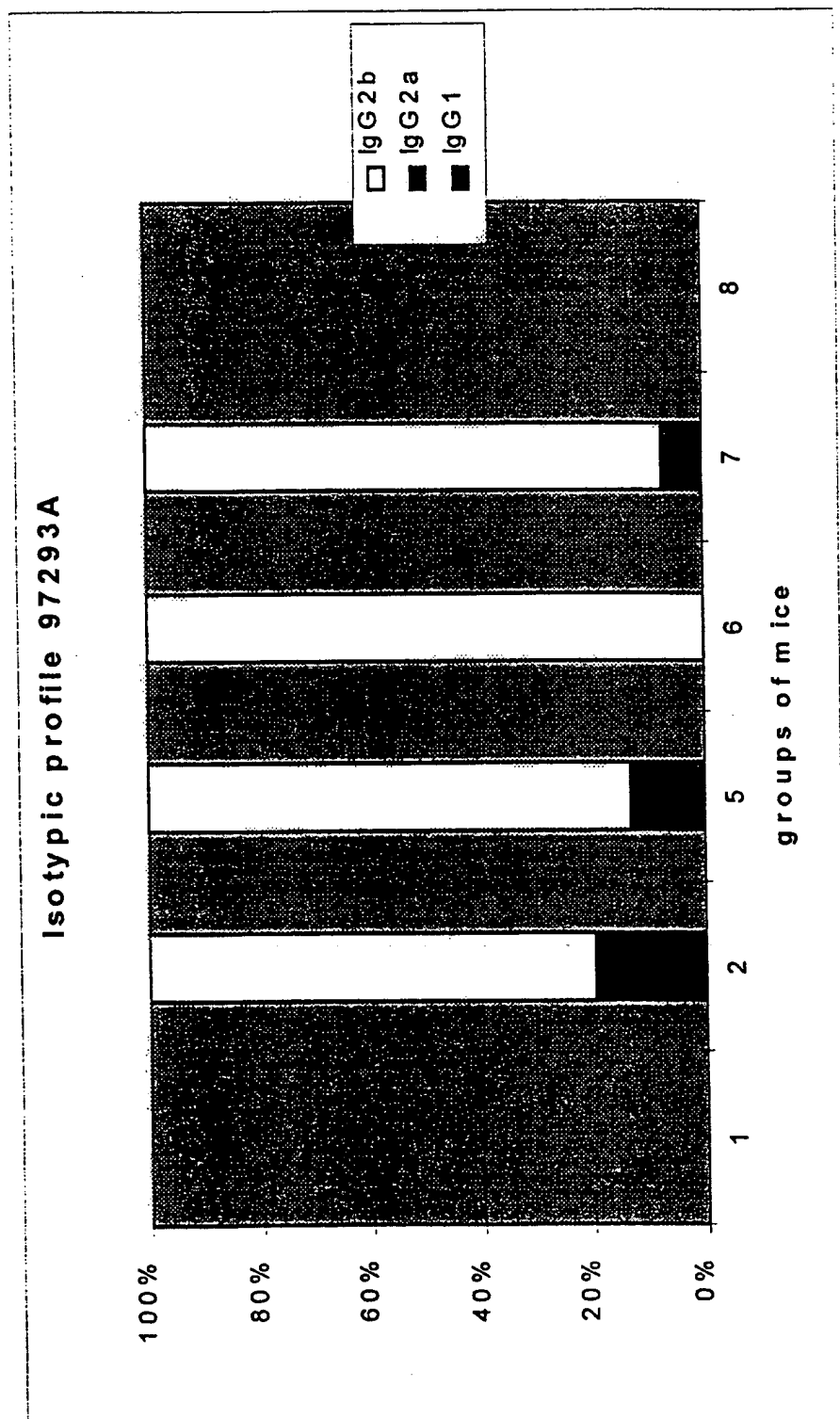


Fig. 4

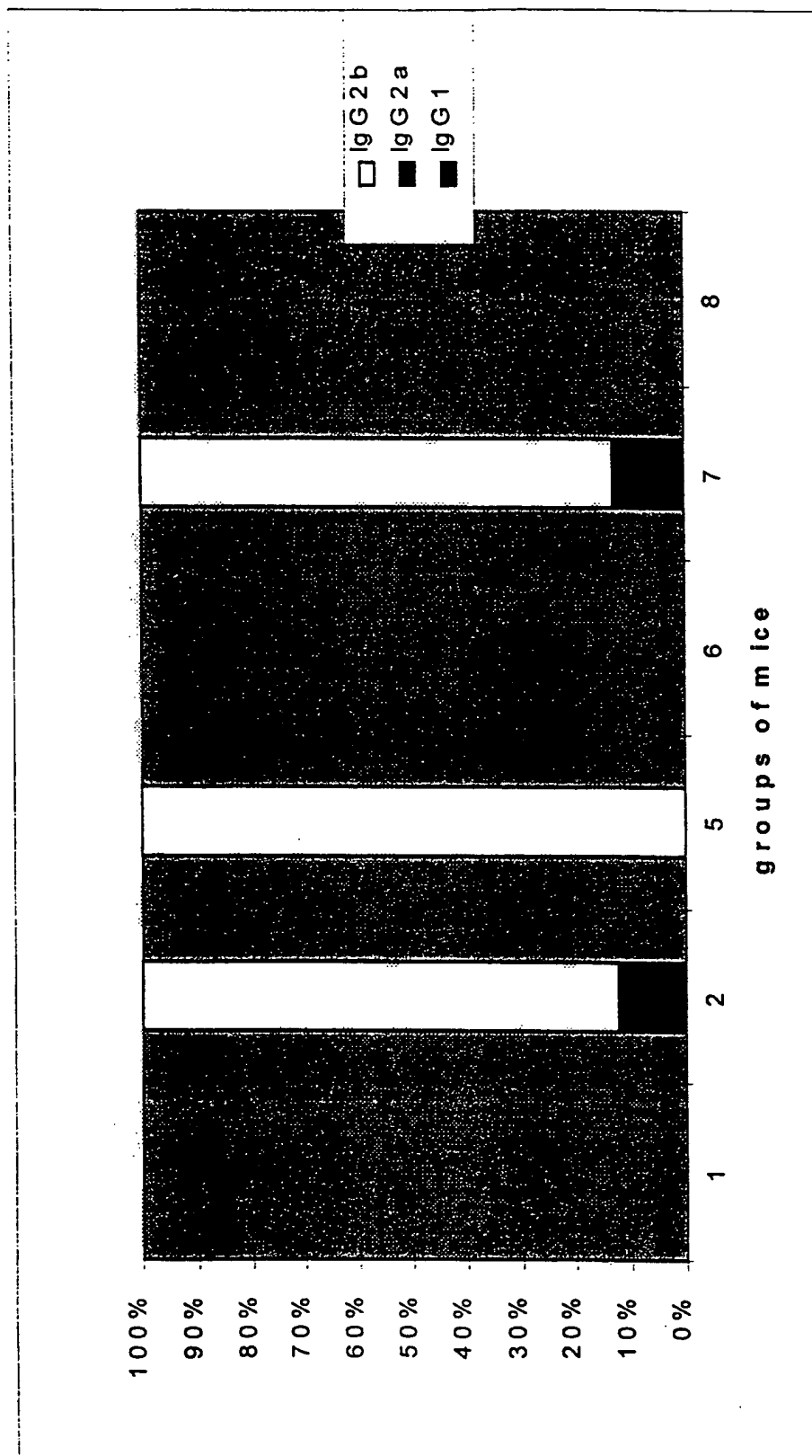


Fig. 5

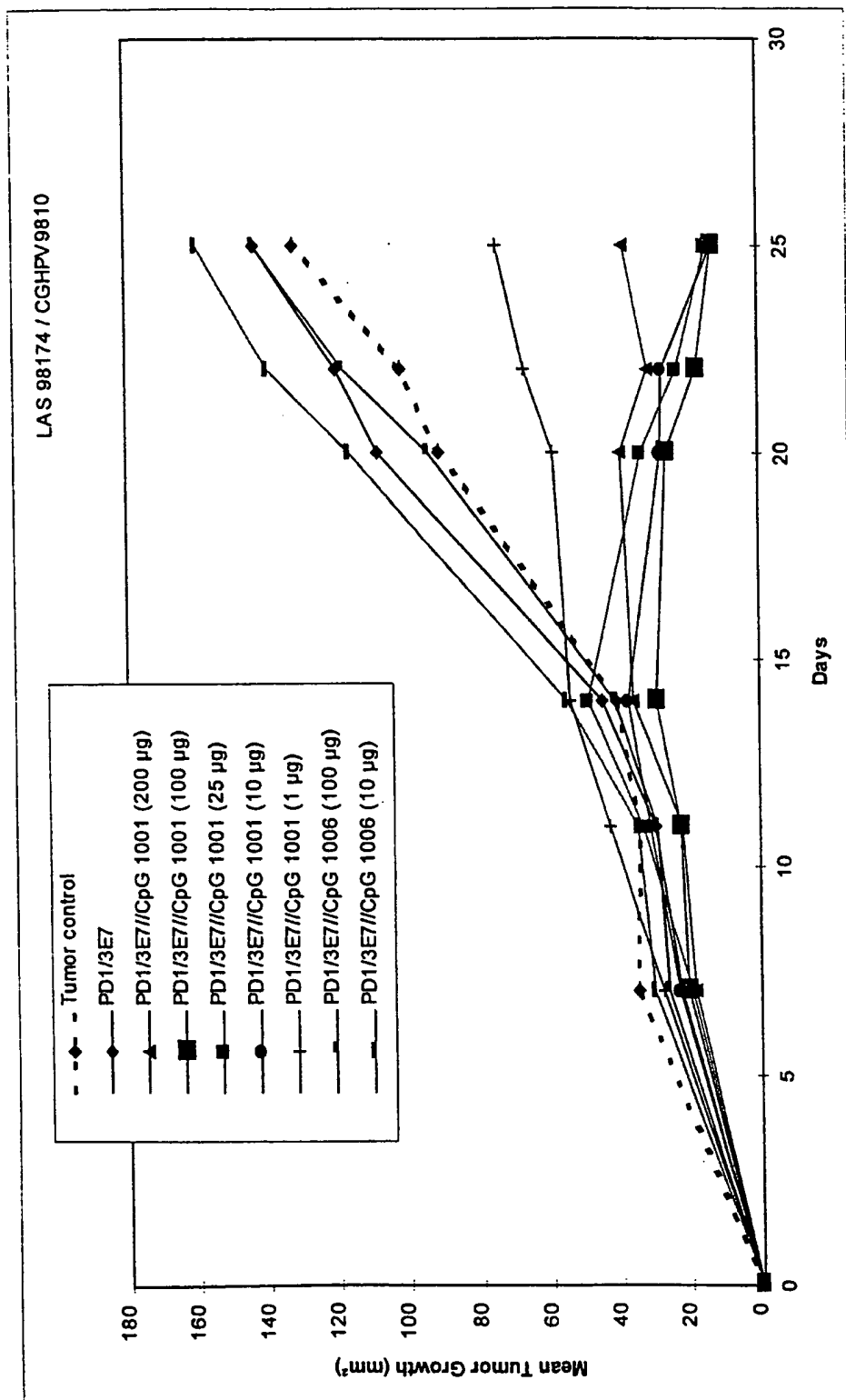
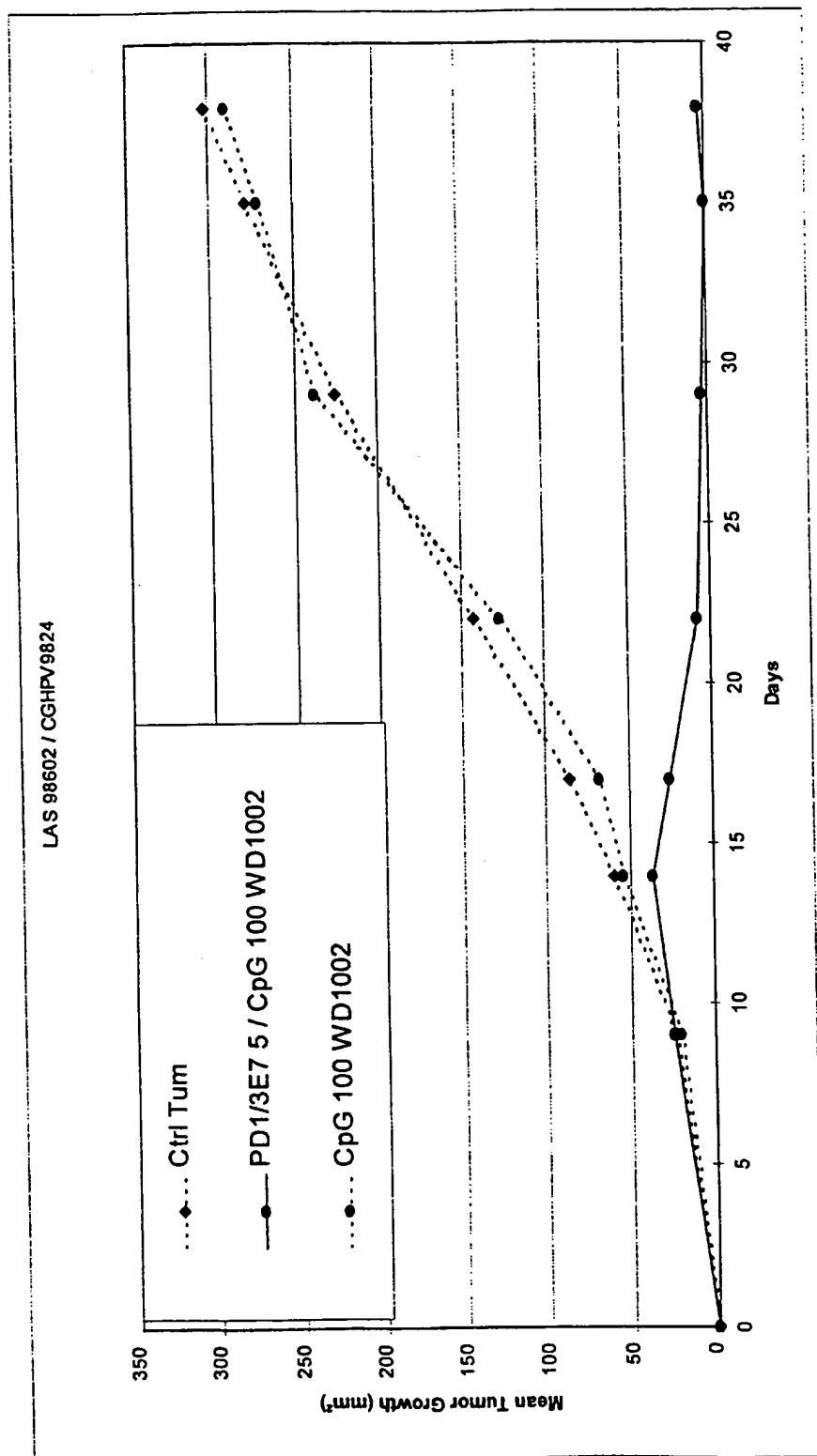


Fig. 6



SEQUENCE LISTING

(1) GENERAL INFORMATION

5

(i) APPLICANT: BRUCK, CLAUDINE

(ii) TITLE OF THE INVENTION: VACCINE

10

(iii) NUMBER OF SEQUENCES: 23

(iv) CORRESPONDENCE ADDRESS:

15

(A) ADDRESSEE: SmithKline Beecham

(B) STREET: 2 New Horizons Court, Great West Road, B

(C) CITY: Middx

(D) STATE:

(E) COUNTRY: UK

(F) ZIP: TW8 9EP

20

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette

(B) COMPUTER: IBM Compatible

(C) OPERATING SYSTEM: DOS

25

(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

30

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

35

(viii) ATTORNEY/AGENT INFORMATION:

40

(A) NAME: Dalton, Marcus J

(B) REGISTRATION NUMBER:

(C) REFERENCE/DOCKET NUMBER: B45124

(ix) TELECOMMUNICATION INFORMATION:

45

(A) TELEPHONE: 0181 9756348

(B) TELEFAX: 0181 9756177

(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

50

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 220 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

55

(D) TOPOLOGY: linear

Protein D 1/3 E7 his

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

60

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1 5 10 15

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20 25 30

Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp

65

35 40 45

Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val

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100      105      110
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10 Gln Pro Glu Thr Thr Asp Leu Tyr Cys Tyr Glu Gln Leu Asn Asp Ser
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15      165      170      175
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 663 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 Protein D 1/3 E7 his

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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45 360
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55 660
TAA
663

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(2) INFORMATION FOR SEQ ID NO:3:

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(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 822 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 Protein D 1/3 E6 His/HPV 16

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(2) INFORMATION FOR SEQ ID NO:4:

- 35 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 274 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 40 Protein D 1/3 E6 His/HPV 16

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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 35 40 45
 50 Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
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 Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
 65 70 75 80
 Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
 85 90 95
 55 Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
 100 105 110
 Ala Met Phe Gln Asp Pro Gln Glu Arg Pro Arg Lys Leu Pro Gln Leu
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 130 135 140
 Tyr Cys Lys Gln Gln Leu Leu Arg Arg Glu Val Tyr Asp Phe Ala Phe
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 Arg Asp Leu Cys Ile Val Tyr Arg Asp Gly Asn Pro Tyr Ala Val Cys
 165 170 175
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(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1116 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 Protein D 1/3 E6/E7/ HPV16

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

25 ATGGATCCAA GCAGCCATTC ATCAAATATG GCGAATACCC AAATGAAATC AGACAAAATC
 60 ATTATTGCTC ACCGTGGTGC TAGCGGTTAT TTACCAGAGC ATACGTTAGA ATCTAAAGCA
 120 CTTGCGTTTG CACAACAGGC TGATTATTTA GAGCAAGATT TAGCAATGAC TAAGGATGGT
 30 180 CGTTTAGTGG TTATTCACGA TCACTTTTGA GATGGCTTGA CTGATGTTGC GAAAAAATTC
 240 CCACATCGTC ATCGTAAAGA TGGCCGTAC TATGTCATCG ACTTTACCTT AAAAGAAATT
 35 300 CAAAGTTTAG AAATGACAGA AAACCTTGAA ACCATGGCCA TGTTTCAGGA CCCACAGGAG
 360 CGACCCAGAA AGTTACCACA GTTATGCACA GAGCTGCAAA CAACTATACA TGATATAATA
 420 TTAGAATGTG TGTACTGCAA GCAACAGTTA CTGCGACGTG AGGTATATGA CTTTGCTTTT
 40 480 CGGGATTAT GCATAGTATA TAGAGATGGG AATCCATATG CTGTATGTGA TAAATGTTTA
 540 AAGTTTATT CTAAAATTAG TGAGTATAGA CATTATTGTT ATAGTTTGTA TGGAACAACA
 45 600 TTAGAACAGC AATACAACAA ACCGTTGTGT GATTTGTTAA TTAGGTGTAT TAACTGTCAA
 660 AAGCCACTGT GTCCTGAAGA AAAGCAAAGA CATCTGGACA AAAAGCAAAG ATTCCATAAT
 720 ATAAGGGGTC GGTGGACCGG TCGATGTATG TCTTGTTGCA GATCATCAAG AACACGTAGA
 50 780 GAAACCCAGC TGATGCATGG AGATACACCT ACATTGCATG AATATATGTT AGATTGCAA
 840 CCAGAGACAA CTGATCTCTA CTGTTATGAG CAATTAAATG ACAGCTCAGA GGAGGAGGAT
 55 900 GAAATAGATG GTCCAGCTGG ACAAGCAGAA CCGGACAGAG CCCATTACAA TATTGTAACC
 960 TTTTGTTGCA AGTGTGACTC TACGCTTCGG TTGTGCGTAC AAAGCACACA CGTAGACATT
 1020 CGTACTTTGG AAGACCTGTT AATGGGCACA CTAGGAATTG TGTGCCCCAT CTGTTCTCAG
 60 1080 AAACCAACTA GTGGCCACCA TCACCATCAC CATTAA
 1116

65 (2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 372 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

Protein D 1/3 E6/E7/ HPV16

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```

10  Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
    1           5           10           15
    Ser Asp Lys Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
        20           25           30
    Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
    15  35           40           45
    Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
        50           55           60
    Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
    65  65           70           75           80
    Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
    20  85           90           95
    Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
        100          105          110
    Ala Met Phe Gln Asp Pro Gln Glu Arg Pro Arg Lys Leu Pro Gln Leu
    25  115          120          125
    Cys Thr Glu Leu Gln Thr Thr Ile His Asp Ile Ile Leu Glu Cys Val
    130          135          140
    Tyr Cys Lys Gln Gln Leu Leu Arg Arg Glu Val Tyr Asp Phe Ala Phe
    145          150          155          160
    30  Arg Asp Leu Cys Ile Val Tyr Arg Asp Gly Asn Pro Tyr Ala Val Cys
        165          170          175
    Asp Lys Cys Leu Lys Phe Tyr Ser Lys Ile Ser Glu Tyr Arg His Tyr
    180          185          190
    Cys Tyr Ser Leu Tyr Gly Thr Thr Leu Glu Gln Gln Tyr Asn Lys Pro
    35  195          200          205
    Leu Cys Asp Leu Leu Ile Arg Cys Ile Asn Cys Gln Lys Pro Leu Cys
    210          215          220
    Pro Glu Glu Lys Gln Arg His Leu Asp Lys Lys Gln Arg Phe His Asn
    225          230          235          240
    40  Ile Arg Gly Arg Trp Thr Gly Arg Cys Met Ser Cys Cys Arg Ser Ser
        245          250          255
    Arg Thr Arg Arg Glu Thr Gln Leu Met His Gly Asp Thr Pro Thr Leu
    260          265          270
    His Glu Tyr Met Leu Asp Leu Gln Pro Glu Thr Thr Asp Leu Tyr Cys
    45  275          280          285
    Tyr Glu Gln Leu Asn Asp Ser Ser Glu Glu Glu Asp Glu Ile Asp Gly
    290          295          300
    Pro Ala Gly Gln Ala Glu Pro Asp Arg Ala His Tyr Asn Ile Val Thr
    305          310          315          320
    50  Phe Cys Cys Lys Cys Asp Ser Thr Leu Arg Leu Cys Val Gln Ser Thr
        325          330          335
    His Val Asp Ile Arg Thr Leu Glu Asp Leu Leu Met Gly Thr Leu Gly
    340          345          350
    Ile Val Cys Pro Ile Cys Ser Gln Lys Pro Thr Ser Gly His His His
    55  355          360          365
    His His His
        370

```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 663 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

Protein D 1/3 E7 mutated HPV 16

210

215

220

(2) INFORMATION FOR SEQ ID NO:9:

5 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 879 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

10 CLYTA E6 His HPV 16

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

15 ATGAAAGGGG GAATTGTACA TTCAGACGGC TCTTATCCAA AAGACAAGTT TGAGAAAATC
 60 AATGGCACTT GGTACTACTT TGACAGTTCA GGCTATATGC TTGCAGACCG CTGGAGGAAG
 120 CACACAGACG GCAACTGGTA CTGGTTCGAC AACTCAGGCG AAATGGCTAC AGGCTGGAAG
 180 AAAATCGCTG ATAAGTGGTA CTATTTCAAC GAAGAAGGTG CCATGAAGAC AGGCTGGGTC
 20 240 AAGTACAAGG ACACTTGGTA CTACTTAGAC GCTAAAGAAG GCGCCATGGT ATCAATGCC
 300 TTTATCCAGT CAGCGGACGG AACAGGCTGG TACTACCTCA AACCAGACGG AACACTGGCA
 25 360 GACAGGCCAG AATTGGCCAG CATGCTGGAC ATGGCCATGT TTCAGGACCC ACAGGAGCGA
 420 CCCAGAAAGT TACCACAGTT ATGCACAGAG CTGCAAACAA CTATACATGA TATAATATTA
 480 GAATGTGTGT ACTGCAAGCA ACAGTTACTG CGACGTGAGG TATATGACTT TGCTTTTCGG
 540 GATTATGCA TAGTATATAG AGATGGGAAT CCATATGCTG TATGTGATAA ATGTTTAAAG
 600 TTTTATTCTA AAATTAGTGA GTATAGACAT TATTGTTATA GTTTGTATGG AACAACTTA
 35 660 GAACAGCAAT ACAACAAACC GTTGTGTGAT TTGTTAATTA GGTGTATTAA CTGTCAAAAG
 720 CCACTGTGTC CTGAAGAAAA GCAAAGACAT CTGGACAAAA AGCAAAGATT CCATAATATA
 780 AGGGGTCGGT GGACCGGTCG ATGTATGTCT TGTTGCAGAT CATCAAGAAC ACGTAGAGAA
 40 840 ACCCAGCTGA CTAGTGGCCA CCATCACCAT CACCATTAA
 879

45 (2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 293 amino acids

(B) TYPE: amino acid

50 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

CLYTA E6 His HPV 16

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

55 Met Lys Gly Gly Ile Val His Ser Asp Gly Ser Tyr Pro Lys Asp Lys
 1 5 10 15
 Phe Glu Lys Ile Asn Gly Thr Trp Tyr Tyr Phe Asp Ser Ser Gly Tyr
 20 25 30
 60 Met Leu Ala Asp Arg Trp Arg Lys His Thr Asp Gly Asn Trp Tyr Trp
 35 40 45
 Phe Asp Asn Ser Gly Glu Met Ala Thr Gly Trp Lys Lys Ile Ala Asp
 50 55 60
 Lys Trp Tyr Tyr Phe Asn Glu Glu Gly Ala Met Lys Thr Gly Trp Val
 65 65 70 75 80
 Lys Tyr Lys Asp Thr Trp Tyr Tyr Leu Asp Ala Lys Glu Gly Ala Met

					85					90					95	
	Val	Ser	Asn	Ala	Phe	Ile	Gln	Ser	Ala	Asp	Gly	Thr	Gly	Trp	Tyr	Tyr
				100					105					110		
5	Leu	Lys	Pro	Asp	Gly	Thr	Leu	Ala	Asp	Arg	Pro	Glu	Leu	Ala	Ser	Met
			115					120					125			
	Leu	Asp	Met	Ala	Met	Phe	Gln	Asp	Pro	Gln	Glu	Arg	Pro	Arg	Lys	Leu
		130					135					140				
	Pro	Gln	Leu	Cys	Thr	Glu	Leu	Gln	Thr	Thr	Ile	His	Asp	Ile	Ile	Leu
		145				150					155				160	
10	Glu	Cys	Val	Tyr	Cys	Lys	Gln	Gln	Leu	Leu	Arg	Arg	Glu	Val	Tyr	Asp
					165					170					175	
	Phe	Ala	Phe	Arg	Asp	Leu	Cys	Ile	Val	Tyr	Arg	Asp	Gly	Asn	Pro	Tyr
				180					185					190		
15	Ala	Val	Cys	Asp	Lys	Cys	Leu	Lys	Phe	Tyr	Ser	Lys	Ile	Ser	Glu	Tyr
			195					200					205			
	Arg	His	Tyr	Cys	Tyr	Ser	Leu	Tyr	Gly	Thr	Thr	Leu	Glu	Gln	Gln	Tyr
		210					215					220				
	Asn	Lys	Pro	Leu	Cys	Asp	Leu	Leu	Ile	Arg	Cys	Ile	Asn	Cys	Gln	Lys
		225				230					235				240	
20	Pro	Leu	Cys	Pro	Glu	Lys	Gln	Arg	His	Leu	Asp	Lys	Lys	Gln	Arg	
					245					250				255		
	Phe	His	Asn	Ile	Arg	Gly	Arg	Trp	Thr	Gly	Arg	Cys	Met	Ser	Cys	Cys
				260					265					270		
25	Arg	Ser	Ser	Arg	Thr	Arg	Arg	Glu	Thr	Gln	Leu	Thr	Ser	Gly	His	His
			275				280						285			
	His	His	His	His												
				290												

(2) INFORMATION FOR SEQ ID NO:11:

30

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 720 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

35

(D) TOPOLOGY: linear

CLYTA E7 HIS HPV 16

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

40	ATGAAAGGGG GAATTGTACA TTCAGACGGC TCTTATCCAA AAGACAAGTT TGAGAAAATC
60	AATGGCACTT GGTACTACTT TGACAGTTCA GGCTATATGC TTGCAGACCG CTGGAGGAAG
	120 CACACAGACG GCAACTGGTA CTGGTTCGAC AACTCAGGCG AAATGGCTAC AGGCTGGAAG
45	180 AAAATCGCTG ATAAGTGGTA CTATTTCAAC GAAGAAGGTG CCATGAAGAC AGGCTGGGTC
	240 AAGTACAAGG AACTTGGTA CTAATTAGAC GCTAAAGAAG GCGCCATGGT ATCAAATGCC
50	300 TTTATCCAGT CAGCGGACGG AACAGGCTGG TACTACCTCA AACCAGACGG AACACTGGCA
	360 GACAGGCCAG AATTGGCCAG CATGCTGGAC ATGGCCATGC ATGGAGATAC ACCTACATTG
	420 CATGAATATA TGTTAGATTT GCAACCAGAG ACAACTGATC TCTACTGTTA TGAGCAATTA
55	480 AATGACAGCT CAGAGGAGGA GGATGAAATA GATGGTCCAG CTGGACAAGC AGAACCGGAC
	540 AGAGCCCATT ACAATATTGT AACCTTTTGT TGCAAGTGTG ACTCTACGCT TCGGTTGTGC
	600 GTACAAAGCA CACACGTAGA CATTCTGACT TTGGAAGACC TGTTAATGGG CACACTAGGA
60	660 ATTGTGTGCC CCATCTGTTC TCAGAAACCA ACTAGTGGCC ACCATCACCA TCACCATTAA
	720

65

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 240 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 CLYTA E7 HIS HPV 16

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

10 Met Lys Gly Gly Ile Val His Ser Asp Gly Ser Tyr Pro Lys Asp Lys
 1 5 10 15
 Phe Glu Lys Ile Asn Gly Thr Trp Tyr Phe Asp Ser Ser Gly Tyr
 20 25 30
 Met Leu Ala Asp Arg Trp Arg Lys His Thr Asp Gly Asn Trp Tyr Trp
 35 40 45
 15 Phe Asp Asn Ser Gly Glu Met Ala Thr Gly Trp Lys Lys Ile Ala Asp
 50 55 60
 Lys Trp Tyr Tyr Phe Asn Glu Glu Gly Ala Met Lys Thr Gly Trp Val
 65 70 75 80
 20 Lys Tyr Lys Asp Thr Trp Tyr Tyr Leu Asp Ala Lys Glu Gly Ala Met
 85 90 95
 Val Ser Asn Ala Phe Ile Gln Ser Ala Asp Gly Thr Gly Trp Tyr Tyr
 100 105 110
 Leu Lys Pro Asp Gly Thr Leu Ala Asp Arg Pro Glu Leu Ala Ser Met
 115 120 125
 25 Leu Asp Met Ala Met His Gly Asp Thr Pro Thr Leu His Glu Tyr Met
 130 135 140
 Leu Asp Leu Gln Pro Glu Thr Thr Asp Leu Tyr Cys Tyr Glu Gln Leu
 145 150 155 160
 30 Asn Asp Ser Ser Glu Glu Asp Glu Ile Asp Gly Pro Ala Gly Gln
 165 170 175
 Ala Glu Pro Asp Arg Ala His Tyr Asn Ile Val Thr Phe Cys Cys Lys
 180 185 190
 Cys Asp Ser Thr Leu Arg Leu Cys Val Gln Ser Thr His Val Asp Ile
 195 200 205
 35 Arg Thr Leu Glu Asp Leu Leu Met Gly Thr Leu Gly Ile Val Cys Pro
 210 215 220
 Ile Cys Ser Gln Lys Pro Thr Ser Gly His His His His His His
 225 230 235

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1173 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 CLYTA E6E7 His HPV16

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ATGAAAGGGG GAATTGTACA TTCAGACGGC TCTTATCCAA AAGACAAGTT TGAGAAAATC
 60
 AATGGCACTT GGTACTACTT TGACAGTTCA GGCTATATGC TTGCAGACCG CTGGAGGAAG
 120
 CACACAGACG GCAACTGGTA CTGGTTCGAC AACTCAGGCG AAATGGCTAC AGGCTGGAAG
 180
 AAAATCGCTG ATAAGTGGTA CTATTTCAAC GAAGAAGGTG CCATGAAGAC AGGCTGGGTC
 240
 AAGTACAAGG ACACTTGGTA CTA CTACTTAGAC GCTAAAGAAG GCGCCATGGT ATCAAATGCC
 300
 TTTATCCAGT CAGCGGACGG AACAGGCTGG TACTACCTCA AACCAGACGG AACACTGGCA
 360
 GACAGGCCAG AATTGGCCAG CATGCTGGAC ATGGCCATGT TTCAGGACCC ACAGGAGCGA
 420

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      CCCAGAAAGT TACCACAGTT ATGCACAGAG CTGCAAACAA CTATACATGA TATAATATTA
480
      GAATGTGTGT ACTGCAAGCA ACAGTTACTG CGACGTGAGG TATATGACTT TGCTTTTCGG
540
5      GATTATGCA TAGTATATAG AGATGGGAAT CCATATGCTG TATGTGATAA ATGTTTAAAG
600
      TTTTATTCTA AAATTAGTGA GTATAGACAT TATTGTTATA GTTTGTATGG AACACATTA
660
      GAACAGCAAT ACAACAAACC GTTGTGTGAT TTGTTAATTA GGTGTATTAA CTGTCAAAAG
10     720
      CCACTGTGTC CTGAAGAAAA GCAAAGACAT CTGGACAAAA AGCAAAGATT CCATAATATA
780
      AGGGGTCGGT GGACCGGTCG ATGTATGTCT TGTTCAGAT CATCAAGAAC ACGTAGAGAA
840
15     ACCCAGCTGA TGCATGGAGA TACACCTACA TTGCATGAAT ATATGTTAGA TTTGCAACCA
900
      GAGACAACTG ATCTCTACTG TTATGAGCAA TTAAATGACA GCTCAGAGGA GGAGGATGAA
960
      ATAGATGGTC CAGCTGGACA AGCAGAACCG GACAGAGCCC ATTACAATAT TGTAACCTTT
20     1020
      TGTGCAAGT GTGACTCTAC GCTTCGGTTG TCGGTACAAA GCACACACGT AGACATTCGT
1080
      ACTTTGGAAG ACCTGTTAAT GGGCACACTA GGAATTGTGT GCCCCATCTG TTCTCAGAAA
1140
25     CCAACTAGTG GCCACCATCA CCATCACCAT TAA
1173

```

(2) INFORMATION FOR SEQ ID NO:14:

```

30     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 391 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
35     CLYTA E6E7 His HPV16

```

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```

40     Met Lys Gly Gly Ile Val His Ser Asp Gly Ser Tyr Pro Lys Asp Lys
          1           5           10           15
      Phe Glu Lys Ile Asn Gly Thr Trp Tyr Tyr Phe Asp Ser Ser Gly Tyr
          20           25           30
      Met Leu Ala Asp Arg Trp Arg Lys His Thr Asp Gly Asn Trp Tyr Trp
          35           40           45
45     Phe Asp Asn Ser Gly Glu Met Ala Thr Gly Trp Lys Lys Ile Ala Asp
          50           55           60
      Lys Trp Tyr Tyr Phe Asn Glu Glu Gly Ala Met Lys Thr Gly Trp Val
          65           70           75           80
      Lys Tyr Lys Asp Thr Trp Tyr Tyr Leu Asp Ala Lys Glu Gly Ala Met
          85           90           95
50     Val Ser Asn Ala Phe Ile Gln Ser Ala Asp Gly Thr Gly Trp Tyr Tyr
          100          105          110
      Leu Lys Pro Asp Gly Thr Leu Ala Asp Arg Pro Glu Leu Ala Ser Met
          115          120          125
55     Leu Asp Met Ala Met Phe Gln Asp Pro Gln Glu Arg Pro Arg Lys Leu
          130          135          140
      Pro Gln Leu Cys Thr Glu Leu Gln Thr Thr Ile His Asp Ile Ile Leu
          145          150          155          160
      Glu Cys Val Tyr Cys Lys Gln Gln Leu Leu Arg Arg Glu Val Tyr Asp
          165          170          175
60     Phe Ala Phe Arg Asp Leu Cys Ile Val Tyr Arg Asp Gly Asn Pro Tyr
          180          185          190
      Ala Val Cys Asp Lys Cys Leu Lys Phe Tyr Ser Lys Ile Ser Glu Tyr
          195          200          205
65     Arg His Tyr Cys Tyr Ser Leu Tyr Gly Thr Thr Leu Glu Gln Gln Tyr
          210          215          220

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Asn Lys Pro Leu Cys Asp Leu Leu Ile Arg Cys Ile Asn Cys Gln Lys
 225 230 235 240
 Pro Leu Cys Pro Glu Lys Gln Arg His Leu Asp Lys Lys Gln Arg
 245 250 255
 5 Phe His Asn Ile Arg Gly Arg Trp Thr Gly Arg Cys Met Ser Cys Cys
 260 265 270
 Arg Ser Ser Arg Thr Arg Arg Glu Thr Gln Leu Met His Gly Asp Thr
 275 280 285
 10 Pro Thr Leu His Glu Tyr Met Leu Asp Leu Gln Pro Glu Thr Thr Asp
 290 295 300
 Leu Tyr Cys Tyr Glu Gln Leu Asn Asp Ser Ser Glu Glu Glu Asp Glu
 305 310 315 320
 Ile Asp Gly Pro Ala Gly Gln Ala Glu Pro Asp Arg Ala His Tyr Asn
 325 330 335
 15 Ile Val Thr Phe Cys Cys Lys Cys Asp Ser Thr Leu Arg Leu Cys Val
 340 345 350
 Gln Ser Thr His Val Asp Ile Arg Thr Leu Glu Asp Leu Leu Met Gly
 355 360 365
 20 Thr Leu Gly Ile Val Cys Pro Ile Cys Ser Gln Lys Pro Thr Ser Gly
 370 375 380
 His His His His His His
 385 390

(2) INFORMATION FOR SEQ ID NO:15:

25

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 684 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 Protein D 1/3 E7 his HPV 18

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

35

ATGGATCCAA GCAGCCATTC ATCAAATATG GCGAATACCC AAATGAAATC AGACAAAATC
 60
 ATTATTGCTC ACCGTGGTGC TAGCGGTTAT TTACCAGAGC ATACGTTAGA ATCTAAAGCA
 120
 40 CTGCGTTTG CACAACAGGC TGATTATTTA GAGCAAGATT TAGCAATGAC TAAGGATGGT
 180
 CGTTTAGTGG TTATTCACGA TCACTTTTGA GATGGCTTGA CTGATGTTGC GAAAAAATTC
 240
 CCACATCGTC ATCGTAAAGA TGGCCGTTAC TATGTCATCG ACTTTACCTT AAAAGAAATT
 300
 45 CAAAGTTTAG AAATGACAGA AAACTTTGAA ACCATGGCCA TGCATGGACC TAAGGCAACA
 360
 TTGCAAGACA TTGTATTGCA TTTAGAGCCC CAAAATGAAA TTCCGTTGA CCTTCTATGT
 420
 50 CACGAGCAAT TAAGCGACTC AGAGGAAGAA AACGATGAAA TAGATGAAGT TAATCATCAA
 480
 CATTACCAG CCCGACGAGC CGAACCACAA CGTCACACAA TGTGTGTAT GTGTTGTAAG
 540
 TGTGAAGCCA GAATTGAGCT AGTAGTAGAA AGCTCAGCAG ACGACCTTCG AGCATTCCAG
 600
 55 CAGCTGTTTC TGAACACCCT GTCCTTTGTG TGTCCGTGGT GTGCATCCCA GCAGACTAGT
 660
 GGCCACCATC ACCATCACCA TTAA
 684

60

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 228 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single

65

(D) TOPOLOGY: linear
Protein D 1/3 E7 his HPV 18

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```

5      Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
      1      5      10      15
      Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
      20      25      30
10     Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Ala Asp
      35      40      45
      Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
      50      55      60
      Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
15     65      70      75      80
      Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
      85      90      95
      Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
      100     105     110
20     Ala Met His Gly Pro Lys Ala Thr Leu Gln Asp Ile Val Leu His Leu
      115     120     125
      Glu Pro Gln Asn Glu Ile Pro Val Asp Leu Leu Cys His Glu Gln Leu
      130     135     140
      Ser Asp Ser Glu Glu Glu Asn Asp Glu Ile Asp Glu Val Asn His Gln
25     145     150     155     160
      His Leu Pro Ala Arg Arg Ala Glu Pro Gln Arg His Thr Met Leu Cys
      165     170     175
      Met Cys Cys Lys Cys Glu Ala Arg Ile Glu Leu Val Val Glu Ser Ser
      180     185     190
30     Ala Asp Asp Leu Arg Ala Phe Gln Gln Leu Phe Leu Asn Thr Leu Ser
      195     200     205
      Phe Val Cys Pro Trp Cys Ala Ser Gln Gln Thr Ser Gly His His His
      210     215     220
      His His His
35     225

```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

```

40     (A) LENGTH: 110 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear
      Thioredoxin
45

```

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```

      Met Ser Asp Lys Ile Ile His Leu Thr Asp Asp Ser Phe Asp Thr Asp
      1      5      10      15
50     Val Leu Lys Ala Asp Gly Ala Ile Leu Val Asp Phe Trp Ala Glu Trp
      20      25      30
      Cys Gly Pro Cys Lys Met Ile Ala Pro Ile Leu Asp Glu Ile Ala Asp
      35      40      45
      Glu Tyr Gln Gly Lys Leu Thr Val Ala Lys Leu Asn Ile Asp Gln Asn
55     50      55      60
      Pro Gly Thr Ala Pro Lys Tyr Gly Ile Arg Gly Ile Pro Thr Leu Leu
      65      70      75      80
      Leu Phe Lys Asn Gly Glu Val Ala Ala Thr Lys Val Gly Ala Leu Ser
      85      90      95
60     Lys Gly Gln Leu Lys Glu Phe Leu Asp Ala Asn Leu Ala
      100     105

```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

```

65     (A) LENGTH: 684 base pairs

```

(B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 Protein D 1/3 E7 mutated HPV 18

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

ATGGATCCAA GCAGCCATTC ATCAAATATG GCGAATACCC AAATGAAATC AGACAAAATC
 60
 10 ATTATTGCTC ACCGTGGTGC TAGCGGTTAT TTACCAGAGC ATACGTTAGA ATCTAAAGCA
 120
 CTTGCGTTTG CACAACAGGC TGATTATTTA GAGCAAGATT TAGCAATGAC TAAGGATGGT
 180
 CGTTTAGTGG TTATTCACGA TCACTTTTTA GATGGCTTGA CTGATGTTGC GAAAAAATTC
 15 240
 CCACATCGTC ATCGTAAAGA TGGCCGTTAC TATGTCATCG ACTTTACCTT AAAAGAAATT
 300
 CAAAGTTTAG AAATGACAGA AAACTTTGAA ACCATGGCCA TGCATGGACC TAAGGCAACA
 360
 20 TTGCAAGACA TTGTATTGCA TTTAGAGCCC CAAAATGAAA TTCCGGTTGA CCTTCTAGGT
 420
 CACCAGCAAT TAAGCGACTC AGAGGAAGAA AACGATGAAA TAGATGGAGT TAATCATCAA
 480
 CATTACCAG CCCGACGAGC CGAACCACAA CGTCACACAA TGTTGTGTAT GTGTTGTAAG
 25 540
 TGTGAAGCCA GAATTGAGCT AGTAGTAGAA AGCTCAGCAG ACGACCTTCG AGCATTCCAG
 600
 CAGCTGTTTC TGAACACCCT GTCCTTTGTG TGTCCGTGGT GTGCATCCCA GCAGACTAGT
 660
 30 GGCCACCATC ACCATCACCA TTAA
 684

(2) INFORMATION FOR SEQ ID NO:19:

35

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 228 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

40

Protein D 1/3 E7 mutated HPV 18

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

45 Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
 1 5 10 15
 Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
 20 25 30
 Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Ala Asp
 35 40 45
 50 Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
 50 55 60
 Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
 65 70 75 80
 55 Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
 85 90 95
 Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
 100 105 110
 Ala Met His Gly Pro Lys Ala Thr Leu Gln Asp Ile Val Leu His Leu
 115 120 125
 60 Glu Pro Gln Asn Glu Ile Pro Val Asp Leu Leu Gly His Gln Gln Leu
 130 135 140
 Ser Asp Ser Glu Glu Glu Asn Asp Glu Ile Asp Gly Val Asn His Gln
 145 150 155 160
 65 His Leu Pro Ala Arg Arg Ala Glu Pro Gln Arg His Thr Met Leu Cys
 165 170 175

[illegible]

10 (2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 837 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

Protein D 1/3 E6 - His HPV 18

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

20	ATGGATCCAA	GCAGCCATTC	ATCAAATATG	GCGAATACCC	AAATGAAATC	AGACAAAATC
60	ATTATTGCTC	ACCGTGGTGC	TAGCGGTTAT	TTACCAGAGC	ATACGTTAGA	ATCTAAAGCA
120	CTTGCGTTTG	CACAACAGGC	TGATTATTTA	GAGCAAGATT	TAGCAATGAC	TAAGGATGGT
25	CGTTTAGTGG	TTATTCACGA	TCACTTTTTA	GATGGCTTGA	CTGATGTTGC	GAAAAAATTC
180	CCACATCGTC	ATCGTAAAGA	TGGCCGTTAC	TATGTCATCG	ACTTTACCTT	AAAAGAAATT
240	CAAAGTTTAG	AAATGACAGA	AAACTTTGAA	ACCATGGCGC	GCTTTGAGGA	TCCAACACGG
30	CGACCCTACA	AGCTACCTGA	TCTGTGCACG	GAACTGAACA	CTTCACTGCA	AGACATAGAA
360	ATAACCTGTG	TATATTGCAA	GACAGTATTG	GAACTTACAG	AGGTATTTGA	ATTTGCATTT
420	AAAGATTTAT	TTGTGGTGTA	TAGAGACAGT	ATACCGCATG	CTGCATGCCA	TAAATGTATA
35	GATTTTTATT	CTAGAATTAG	AGAATTAAGA	CATTATTCAG	ACTCTGTGTA	TGGAGACACA
480	TTGGAAAAAC	TAACCTAACAC	TGGGTTATAC	AATTTATTAA	TAAGGTGCCT	GCGGTGCCAG
540	AAACCGTTGA	ATCCAGCAGA	AAAACCTAGA	CACCTTAATG	AAAAACGACG	ATTTCAACAAC
40	ATAGCTGGGC	ACTATAGAGG	CCAGTGCCAT	TCGTGCTGCA	ACCGAGCACG	ACAGGAACGA
600	CTCCAACGAC	GCAGAGAAAC	ACAAGTAACT	AGTGGCCACC	ATCACCATCA	CCATTAA
660						
720						
780						
837						

50 (2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 279 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

Protein D 1/3 E6 - His HPV 18

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

60	Met	Asp	Pro	Ser	Ser	His	Ser	Ser	Asn	Met	Ala	Asn	Thr	Gln	Met	Lys
	1				5				10					15		
	Ser	Asp	Lys	Ile	Ile	Ile	Ala	His	Arg	Gly	Ala	Ser	Gly	Tyr	Leu	Pro
			20					25						30		
65	Glu	His	Thr	Leu	Glu	Ser	Lys	Ala	Leu	Ala	Phe	Ala	Gln	Gln	Ala	Asp
			35					40					45			

Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
 50 55 60
 Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
 65 70 75 80
 5 Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
 85 90 95
 Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
 100 105 110
 Ala Arg Phe Glu Asp Pro Thr Arg Arg Pro Tyr Lys Leu Pro Asp Leu
 10 115 120 125
 Cys Thr Glu Leu Asn Thr Ser Leu Gln Asp Ile Glu Ile Thr Cys Val
 130 135 140
 Tyr Cys Lys Thr Val Leu Glu Leu Thr Glu Val Phe Glu Phe Ala Phe
 145 150 155 160
 15 Lys Asp Leu Phe Val Val Tyr Arg Asp Ser Ile Pro His Ala Ala Cys
 165 170 175
 His Lys Cys Ile Asp Phe Tyr Ser Arg Ile Arg Glu Leu Arg His Tyr
 180 185 190
 Ser Asp Ser Val Tyr Gly Asp Thr Leu Glu Lys Leu Thr Asn Thr Gly
 195 200 205
 20 Leu Tyr Asn Leu Leu Ile Arg Cys Leu Arg Cys Gln Lys Pro Leu Asn
 210 215 220
 Pro Ala Glu Lys Leu Arg His Leu Asn Glu Lys Arg Arg Phe His Asn
 225 230 235 240
 25 Ile Ala Gly His Tyr Arg Gly Gln Cys His Ser Cys Cys Asn Arg Ala
 245 250 255
 Arg Gln Glu Arg Leu Gln Arg Arg Arg Glu Thr Gln Val Thr Ser Gly
 260 265 270
 30 His His His His His His
 275

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 1152 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

- 40 Protein D1/3 E6 E7 His/ HPV 18
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATGGATCCAA GCAGCCATTC ATCAAATATG GCGAATACCC AAATGAAATC AGACAAAATC
 60
 45 ATTATTGCTC ACCGTGGTGC TAGCGGTTAT TTACCAGAGC ATACGTTAGA ATCTAAAGCA
 120
 CTTGCGTTTG CACAACAGGC TGATTATTTA GAGCAAGATT TAGCAATGAC TAAGGATGGT
 180
 50 CGTTTAGTGG TTATTCACGA TCACTTTTTA GATGGCTTGA CTGATGTTGC GAAAAAATTC
 240
 CCACATCGTC ATCGTAAAGA TGGCCGTTAC TATGTCATCG ACTTTACCTT AAAAGAAATT
 300
 CAAAGTTTAG AAATGACAGA AACTTTGAA ACCATGGCGC GCTTTGAGGA TCCAACACGG
 360
 55 CGACCCTACA AGCTACCTGA TCTGTGCACG GAACTGAACA CTTCACTGCA AGACATAGAA
 420
 ATAACCTGTG TATATTGCAA GACAGTATTG GAACTTACAG AGGTATTTGA ATTTGCATT
 480
 AAAGATTTAT TTGTGGTGTA TAGAGACAGT ATACCGCATG CTGCATGCCA TAAATGTATA
 540
 60 GATTTTTATT CTAGAATTAG AGAATTAAGA CATTATTCAG ACTCTGTGTA TGGAGACACA
 600
 TTGGAAAAAC TAACTAACAC TGGGTTATAC AATTATTAA TAAGGTGCCT GCGGTGCCAG
 660
 65 AAACCGTTGA ATCCAGCAGA AAACTTAGA CACCTTAATG AAAAACGACG ATTCACAAC
 720

ATAGCTGGGC ACTATAGAGG CCAGTGCCAT TCGTGCTGCA ACCGAGCAGC ACAGGAACGA
 780
 CTCCAACGAC GCAGAGAAAC ACAAGTAATG CATGGACCTA AGGCAACATT GCAAGACATT
 840
 5 GTATTGCATT TAGAGCCCCA AAATGAAATT CCGGTTGACC TTCTATGTCA CGAGCAATTA
 900
 AGCGACTCAG AGGAAGAAAA CGATGAAATA GATGGAGTTA ATCATCAACA TTTACCAGCC
 960
 CGACGAGCCG AACCACAACG TCACACAATG TTGTGTATGT GTTGTAAAGT TGAAGCCAGA
 10 1020
 ATTGAGCTAG TAGTAGAAAG CTCAGCAGAC GACCTTCGAG CATTCCAGCA GCTGTTTCTG
 1080
 AACACCCTGT CCTTTGTGTG TCCGTGGTGT GCATCCCAGC AGACTAGTGG CCACCATCAC
 1140
 15 CATCACCATT AA
 1152

(2) INFORMATION FOR SEQ ID NO:23:

20 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 384 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

25 Protein D1/3 E6 E7 His/ HPV 18

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

30	Met	Asp	Pro	Ser	Ser	His	Ser	Ser	Asn	Met	Ala	Asn	Thr	Gln	Met	Lys
	1				5				10					15		
	Ser	Asp	Lys	Ile	Ile	Ile	Ala	His	Arg	Gly	Ala	Ser	Gly	Tyr	Leu	Pro
				20					25					30		
	Glu	His	Thr	Leu	Glu	Ser	Lys	Ala	Leu	Ala	Phe	Ala	Gln	Gln	Ala	Asp
				35				40					45			
35	Tyr	Leu	Glu	Gln	Asp	Leu	Ala	Met	Thr	Lys	Asp	Gly	Arg	Leu	Val	Val
	50					55						60				
	Ile	His	Asp	His	Phe	Leu	Asp	Gly	Leu	Thr	Asp	Val	Ala	Lys	Lys	Phe
	65					70					75					80
	Pro	His	Arg	His	Arg	Lys	Asp	Gly	Arg	Tyr	Tyr	Val	Ile	Asp	Phe	Thr
					85					90				95		
40	Leu	Lys	Glu	Ile	Gln	Ser	Leu	Glu	Met	Thr	Glu	Asn	Phe	Glu	Thr	Met
				100					105					110		
	Ala	Arg	Phe	Glu	Asp	Pro	Thr	Arg	Arg	Pro	Tyr	Lys	Leu	Pro	Asp	Leu
				115				120					125			
45	Cys	Thr	Glu	Leu	Asn	Thr	Ser	Leu	Gln	Asp	Ile	Glu	Ile	Thr	Cys	Val
	130					135						140				
	Tyr	Cys	Lys	Thr	Val	Leu	Glu	Leu	Thr	Glu	Val	Phe	Glu	Phe	Ala	Phe
	145					150					155					160
	Lys	Asp	Leu	Phe	Val	Val	Tyr	Arg	Asp	Ser	Ile	Pro	His	Ala	Ala	Cys
					165					170				175		
50	His	Lys	Cys	Ile	Asp	Phe	Tyr	Ser	Arg	Ile	Arg	Glu	Leu	Arg	His	Tyr
				180					185					190		
	Ser	Asp	Ser	Val	Tyr	Gly	Asp	Thr	Leu	Glu	Lys	Leu	Thr	Asn	Thr	Gly
				195				200					205			
55	Leu	Tyr	Asn	Leu	Leu	Ile	Arg	Cys	Leu	Arg	Cys	Gln	Lys	Pro	Leu	Asn
	210					215						220				
	Pro	Ala	Glu	Lys	Leu	Arg	His	Leu	Asn	Glu	Lys	Arg	Arg	Phe	His	Asn
	225					230						235				240
	Ile	Ala	Gly	His	Tyr	Arg	Gly	Gln	Cys	His	Ser	Cys	Cys	Asn	Arg	Ala
				245						250				255		
60	Arg	Gln	Glu	Arg	Leu	Gln	Arg	Arg	Arg	Glu	Thr	Gln	Val	Met	His	Gly
				260					265					270		
	Pro	Lys	Ala	Thr	Leu	Gln	Asp	Ile	Val	Leu	His	Leu	Glu	Pro	Gln	Asn
				275				280					285			
65	Glu	Ile	Pro	Val	Asp	Leu	Leu	Cys	His	Glu	Gln	Leu	Ser	Asp	Ser	Glu
	290					295						300				

	Glu	Glu	Asn	Asp	Glu	Ile	Asp	Gly	Val	Asn	His	Gln	His	Leu	Pro	Ala
	305					310					315					320
	Arg	Arg	Ala	Glu	Pro	Gln	Arg	His	Thr	Met	Leu	Cys	Met	Cys	Cys	Lys
					325					330					335	
5	Cys	Glu	Ala	Arg	Ile	Glu	Leu	Val	Val	Glu	Ser	Ser	Ala	Asp	Asp	Leu
					340				345					350		
	Arg	Ala	Phe	Gln	Gln	Leu	Phe	Leu	Asn	Thr	Leu	Ser	Phe	Val	Cys	Pro
			355					360					365			
	Trp	Cys	Ala	Ser	Gln	Gln	Thr	Ser	Gly	His	His	His	His	His	His	
10		370					375					380				